IDENTIFICATION OF RESIDENT AND CIRCULATING ENDOTHELIAL STEM CELLS

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DEDICATION

To my family, for their love and support.

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Blood vessels and circulating blood contain rare immature endothelial cells that display *in vitro* clonal proliferative potential and *in vivo* vessel forming ability. However their precise location, origin, surface marker and molecular determinants have yet to be precisely defined. In this research body of work, we have identified ABCG2, an ATP binding cassette drug transporter that is expressed by stem cells of many lineages, to label vascular endothelial stem cells (VESC) and circulating endothelial stem cells (CESC). During development, ABCG2 expressing VESC are distributed in arterial, venous, and capillary vessels of multiple tissues including heart, lung, bone marrow and retina. They possess clonal colony forming potential *in vitro* and contribute to the growth of arteries, veins and capillaries *in vivo*. Steady state adult tissues also contain VESC that retain colony forming potential, though their frequency is decreased. In human umbilical vessels, $ABCG2^{+}$ VSEC represent about 1% of umbilical cord vessel EC and showed higher colony forming potential than $ABCG2^{-}$ EC. In addition, CESC that could form EC colonies *in vitro* were identified from neonatal murine peripheral blood. About 30% of CESC were labeled by ABCG2. Lineage tracing experiments using hematopoietic (Flk2Cre) and EC (Tie2ERTCre) specific mice showed CESC were derived from vascular EC, not hematopoietic cells. CESC could participate (at a single cell level) in vessel formation *in vivo* in gel transplantation model. Furthermore, after transplantation, CESC retained secondary

colony forming potential in formed blood vessels. Finally, we show that Abcg2 not only labels, but is also critical for the emergence/maintenance of VESC and the production of CESC. These findings provide a solid foundation to identify the critical roles of endothelial stem cells in vascular development, homeostasis and repair.

Mervin C. Yoder, M.D., Chair

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LIST OF ABBREVIATIONS

4-OHT……. 4 Hydroxyl-Tamoxifen

ABCG2TT…… Abcg2CreERT; ROSATdTomato

BM…….. Bone Marrow

CAC…….. Circulating Angiogenic Cell

CB…… Cord Blood

CEC…….. Circulating Endothelial Cell

cECFC…….. Circulating Endothelial Colony Forming Cell

CESC…….. Circulating Endothelial Stem Cell

CVD…….. Cardiovascular Disease

EC…….. Endothelial Cell

ECFC…… Endothelial Colony Forming Cell

EPC…….. Endothelial Progenitor Cell

Flt3TG…… Flt3cre; mTmG

GFP…….. Green Fluorescence Protein

HSC…….. Hematopoietic Stem Cell

HPC…… Hematopoietic Progenitor Cell

HUAEC…….. Human Umbilical Artery Endothelia Cell

HUVEC…….. Human Umbilical Vein Endothelia Cell

IB4…… Isolectin B4

KO…… Knockout

KSL…… c-kit^{+}Scal1^{+}lineage^{-}

MACS…….. Magnetic Activated Cell Sorting

MNC…….. Mononuclear Cell

MP…….. Main Population

PAD…….. Peripheral Artery Disease

PAH…….. Pulmonary Artery Hypertension

PB…….. Peripheral Blood

PFA…….. Paraformaldehyde

ROSATT…….. RosaTdTomato

RT…….. Room Temperature

SMA…….. Smooth Muscle Actin α

SP…….. Side Population

Tie2TT…….. Tie2CreERT; ROSATdTomato

UEA1…….. Ulex Europaeus Agglutinin I

VESC…….. Vascular Endothelial Stem Cell

WT…….. Wild Type

Chapter I

Introduction

1. The development and heterogeneity of blood vessels.

Function of endothelial cells

Endothelial cells (EC), the cells that line the inner lining of blood vessels, are crucial for the homeostasis of the human body. It has been estimated that the human body contain over 10^{12} EC (Augustin et al., 1994), and EC from a human would cover an area of over 1000m^2 (Augustin et al., 1994). More than just a static barrier between the circulation and tissues, EC play dynamic roles in regulating blood pressure (Dharmashankar and Widlansky, 2010; Sandoo et al., 2010), inflammation (Pober and Sessa, 2007), and the transportation of various molecules like growth factors (Bach, 2015; Bastian et al., 1997), lipids (Goldberg and Bornfeldt, 2013; Hagberg et al., 2013; Hassan et al., 2006), and metabolites (Huang et al., 2012; Kaiser et al., 1993; Mann et al., 2003) to support the growth and maintenance of each organ.

Angiogenesis and vasculogenesis

Owning to the importance of EC, during gastrulation, the cardiovascular system is the first system that is formed in the embryo (Fishman and Stainier, 1994). The development of the vascular system can be separated into two distinct processes: vasculogenesis and angiogenesis (Figure I. 1). Vasculogenesis refers to the process of de novo emergence of blood vessel from EC stem / progenitor cells without pre-existing blood vessels (Patan, 2000, 2004), while angiogenesis describes the

sprouting of EC from pre-existing vessels (Patan, 2000, 2004). Angiogenesis contributes to many processes in animal life including tissue development (Breier, 2000; Chung and Ferrara, 2011; Gariano and Gardner, 2005), tumor growth (Nishida et al., 2006; Nishida, 2005; Weis and Cheresh, 2011) or injury healing (Li et al., 2003; Reinders et al., 2006). It has long been believed that vasculogenesis can only occur during early embryonic development (Cines et al., 1998; Demir et al., 2006), however, the recent discovery of immature circulating EC, which have the ability to form *in vitro* EC colonies and form blood vessels *de novo* upon transplantation into recipient animals (Ingram et al., 2004; Javed et al., 2008), raises the possibility of postnatal vasculogenesis. Other than circulating EC, some blood vessel resident or pluripotent stem cell differentiated progenitor / stem cells of the endothelial lineage have also shown the ability to form *de novo* blood vessels (Alphonse et al., 2015; Fang et al., 2012; Ingram et al., 2005; Prasain et al., 2014; Yu et al., 2016). Additionally, vasculogenesis can also take place during postnatal pathogenic vessel growth like during tumor progression (Baudino et al., 2002; Ribatti et al., 2001).

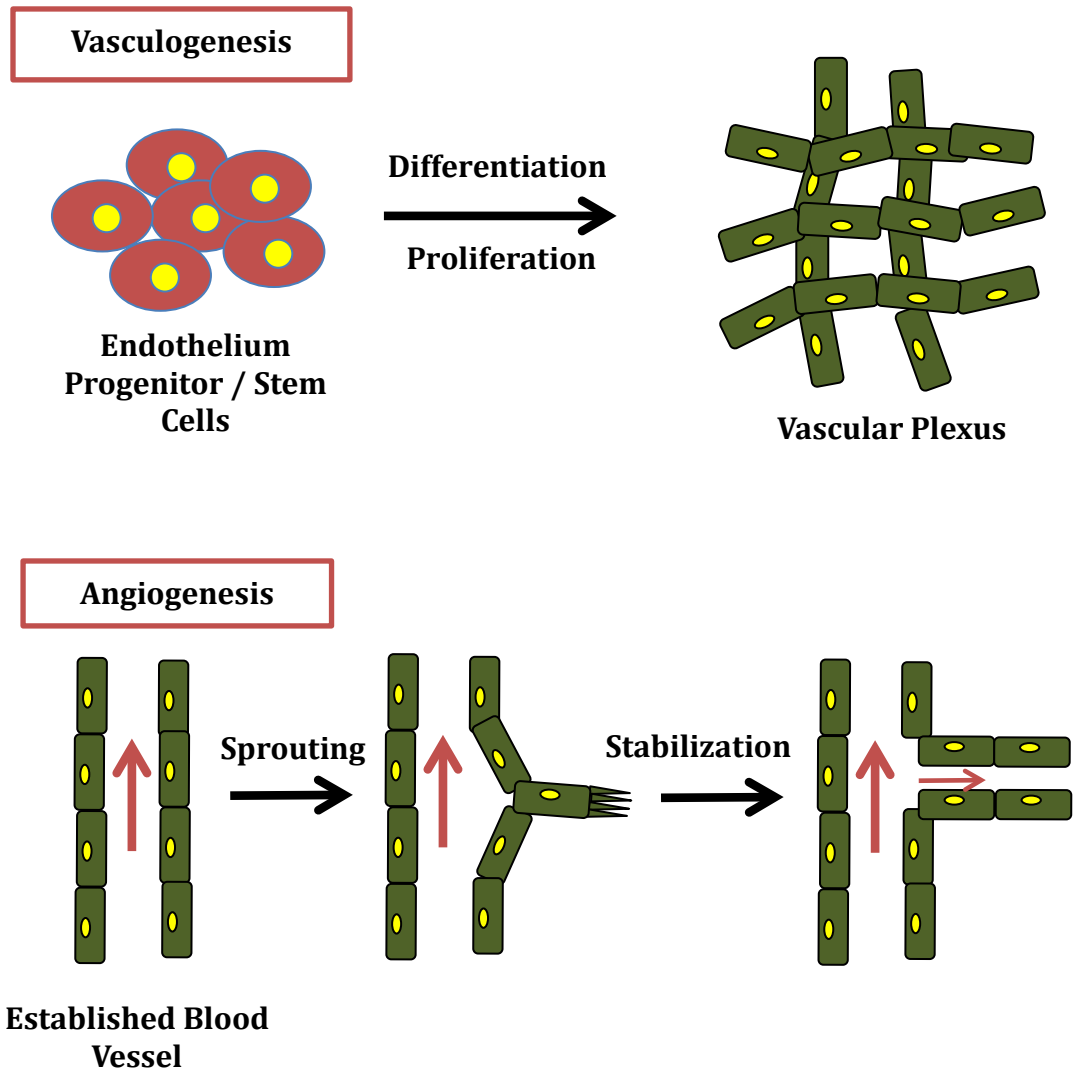


Figure I. 1 Diagram of vasculogenesis (upper panel) and angiogenesis (lower panel)

Early embryonic blood vessel development

The first endothelial precursors, angioblasts, emerge at early gastrulation from mesodermal cells and wrap around clustered primitive hematopoietic cells to form a structure called "blood islands" in the yolk sac (embryonic day [E]7.5 in murine embryos) (Ferkowicz and Yoder, 2005, 2011; Flamme et al., 1997; Palis et al., 1995; Risau and Flamme, 1995). Then the blood islands start to fuse to form lumenized vessel structures and develop into a primary capillary plexus (Palis et al., 1995; Risau and Flamme, 1995). Angioblasts derived from lateral plate mesoderm assemble into early heart tube endocardium and vessels in the brain (Drake and Fleming, 2000; Palis et al., 1995; Risau and Flamme, 1995). Splanchnic mesoderm, somite and presomitic mesoderm contribute to the formation of dorsal aorta EC (Sato, 2013). Umbilical blood vessels are formed by allantoic vasculogenesis (Downs and Harmann, 1997; Drake and Fleming, 2000; Risau and Flamme, 1995). The neovascularization of viscera organs, like lung (Buck et al., 1996; deMello et al., 1997), heart (Luttun and Carmeliet, 2003; Ratajska et al., 2006), kidney (Robert et al., 1998), and liver (Collardeau-Frachon and Scoazec, 2008; Si-Tayeb et al., 2010), are also contributed to by vasculogenesis (Aird, 2007a, b; Pardanaud et al., 1989). After the establishment of initial vascular networks, EC start to branch from existing blood vessels through angiogenesis to support the extension and maintenance of the expanding vasculature in each growing organ (Patan, 2000, 2004).

EC heterogeneity

EC were once thought to be no more than a homogenous sheet of cells that responsd similarly to the stimulation of various growth factors, metabolites, and biomechanical forces. However, recently, more interest and investigation into EC heterogeneity has been developed (Aird, 2007a, b).

AS the first level of EC specificity, EC can be divided into arterial EC, venous EC, and capillary EC. Each type of EC show some unique gene expression patterns and perform different biological functions (Corada et al., 2014; dela Paz and D'Amore, 2009; Kume, 2010). For example, arterial EC express the cell surface molecule EphrinB2 (Corada et al., 2014; dela Paz and D'Amore, 2009; Kume, 2010; Wang et al., 1998), Neuropilin 1 (NrP1)(Alattar et al., 2014), and molecules relate to the Notch signaling pathway including Notch 1 (Liu et al., 2003; Masumura et al., 2009), Notch 4 (Hasan et al., 2017; James et al., 2014; Shutter et al., 2000), Jagged 1 (Lindner et al., 2001; Manderfield et al., 2012), Jagged 2 (Lindner et al., 2001), Hey 1 (Fischer et al., 2004; Hermkens et al., 2015), Hey 2 (Fischer et al., 2004; Hermkens et al., 2015), and Delta-like ligand 4 (Liu et al., 2003; Shutter et al., 2000). Venous EC express EphB4 (dela Paz and D'Amore, 2009; Wang et al., 1998), COUP-TFII (Chen et al., 2012; dela Paz and D'Amore, 2009), and Nrp2 (Sulpice et al., 2008),

The specification of arterial and venous EC is partially governed by hemodynamic force created by blood flow (Corada et al., 2014; dela Paz and D'Amore, 2009;

Masumura et al., 2009; Shutter et al., 2000). The heart starts to beat at E8.5 in the murine embryo to initiate systemic circulation (de Boer et al., 2012; Palis et al., 1995; Savolainen et al., 2009), which brings hematopoietic cells from their origins, yolk sac, placenta or dorsal aorta into the entire vascular system (Lin et al., 2014), and the earliest artery vein specification event proceeds before the establishment of thecirculation (dela Paz and D'Amore, 2009; Jung et al., 2017; Zhong et al., 2001). This suggests that arterial and venous specification is also controlled by some intrinsic or external signaling molecules like Sonic hedgehog (Shh) (Lawson et al., 2002), VEGF (through VEGF-VEGF Receptor 2 pathway) (Covassin et al., 2009; Lawson et al., 2002), Notch (Lawson et al., 2001; Lawson et al., 2002), Sox 7/17/18 (Cermenati et al., 2008; Corada et al., 2013; Hermkens et al., 2015), Klf2 (Dekker et al., 2005; Yamamoto et al., 2015), FoxC1/C2 (Kume et al., 2001) in the arterial EC and COUPTFII (Chen et al., 2012; You et al., 2005) in venous EC specification .

The heterogeneity of vascular EC is also reflected by the functional specificity of EC in each organ. Retina and cerebral EC are linked by tight junctions and thus form the blood-brain barrier and blood-retina barrier (Lippmann et al., 2012; Mohamed et al., 2017; Russ et al., 1998; Stamatovic et al., 2008). Some sinusoidal EC in secretive organs or tissues with absorption or filtration functions like kidney (Satchell and Braet, 2009), liver (Braet and Wisse, 2002; Wisse, 1970), and intestinal villi (Stan et al., 2012) are more fenestrated and thus make the vessels in these organs more permeable.

EC in various tissues are also different by their function of the transportation of biological molecules or inflammatory cells. For example, brain EC highly express Glut1, an efficient glucose transporter, on their surface to provide glucose as the energy source for neurons (Zhao et al., 2015). The surface adhesion molecules, like E- and P-selectin, VCAM-1, and ICAM-1, expressed on the surface of post-capillary venules EC, promote the rolling and attachment of leukocytes to the vascular wall through the binding of co-ligands and eventually induce their transmigration through the vascular wall (Aurrand-Lions et al., 2002; Butcher, 1991; Springer, 1994). Additionally, capillary EC in each organ secrete organ-specific paracrine factors to regulate the development and homeostasis in these tissues (Butler et al., 2010a; Rafii et al., 2016). In the embryo, the development of fetal liver and pancreas are controlled by vascular EC angiocrine factors (Lammert et al., 2001; Matsumoto et al., 2001). In the liver, sinusoidal EC support the proliferation of hepatocytes through Wnt signaling (LeCouter et al., 2003; Wang et al., 2015; Wang et al., 2012), while lung capillary EC derived MMP-14 can promote the regeneration of alveoli epithelial cells after injury (Ding et al., 2015; Ding et al., 2011; Hogan et al., 2014). Additionally, sinusoidal EC also contribute to the maintenance of other lineage-specific stem cells. It is well known that bone marrow microvascular EC play an indispensable role in the hematopoietic stem /progenitor cell niche (Butler et al., 2010b; Kobayashi et al., 2010), and brain sinusoidal EC have been widely used to co-culture neural stem cells *in vitro* as a blood-brain barrier model (Leventhal et al., 1999; Shen et al., 2004) to mimic their *in vivo* role for the maintenance of brain homeostasis (Goldman and Chen, 2011; Licht and Keshet, 2015).

EC's tissue specificity can be identified by the unique gene expression pattern of EC in each tissue (Chi et al., 2003; Nolan et al., 2013). However how these different gene expression profiles are established is still ambiguous. Transcriptome studies showed that various Hox family transcription factors might be responsible for the heterogeneity of vascular EC in each tissue (Douville and Wigle, 2007; Nolan et al., 2013). The tissue microenvironment is known to play an important role in this process, as EC from various origins display the plasticity to adapt to the phenotype and function of local EC after transplantation. Indeed, murine embryonic stem cells derived EC differentiated into liver- or spleen- like EC after incorporating into these two organs in recipient mice (Nolan et al., 2013), and human pluripotent stem cells derived EC could also differentiate into skeletal muscle EC in the hindlimb-ischemia model and retinal EC in a retinopathy model after transplantation (Prasain et al., 2014). Thus, understanding the tissue-specific microenvironment cues for vascular development is crucial for the understanding of tissue EC heterogeneity and will benefit the developing of strategies to rescue injured organs.

In addition to the differences among arterial, venous, and capillary EC and among EC from various tissues, another level of EC heterogeneity lies in differences in the proliferative potential present in different tissues. Each vascular bed contains EC with a hierarchy of proliferative potential, but only some EC progenitor / stem cells in each vascular bed can divide and contribute to the maintenance or growth of blood vessels (Alphonse et al., 2015; Canete et al., 2017; Fang et al., 2012; Huang et al., 2010; Ingram

et al., 2005; Ingram et al., 2004; Kusumbe et al., 2014; Malinverno et al., 2017; Naito et al., 2012; Naito et al., 2016; Nishimura et al., 2015; Patel et al., 2017; Yu et al., 2016), while a majority of EC are mature cells that do not possess proliferative potential. This topic will be discussed in detail in the next section.

2. Vascular endothelial progenitor/stem cells

The turnover of endothelial cells

The majority of EC in the vasculature do not proliferate and the EC turnover rate is generally low at homeostasis (Schwartz and Benditt, 1977), Despite the fact that EC in large arteries are constantly exposed to various types of biomechanical stressors (Davies, 2009; Ilegbusi et al., 1999; Paszkowiak and Dardik, 2003). These biomechanical stresses can inevitably cause injury to EC that line the inner face of blood vessels. At steady state, only 0.1-0.3% of EC in an adult rat aorta proliferate each day (Schwartz and Benditt, 1977). However, when rats at the same age were challenged with experimental renal hypertension, the turnover rate of aortic EC was increased by 10-fold (Schwartz and Benditt, 1977). EC injuries caused by various other cardiovascular diseases, like atherosclerosis (Caplan and Schwartz, 1973; Foteinos et al., 2008; Taylor and Lewis, 1986) and hyperlipidemia (Florentin et al., 1969; Prescott and Muller, 1983), can also induce the increasing of EC turnover rate. Additionally, age is another factor that affects EC turnover. Young animals generally show faster EC proliferation rates than older animals (Schwartz and Benditt, 1977; Taylor and Lewis, 1986).

Some evidence has been presented to show that the turnover of vascular EC is not homogenous. Instead, the intima of blood vessels display some cluster hotspots or foci of EC proliferation (Caplan and Schwartz, 1973; Schwartz and Benditt, 1976,

1977; Wright, 1972), while the majority EC in other areas are not proliferative. While in some cases this can be explained by the increased EC regeneration at areas that are prone to vessel injuries (Caplan and Schwartz, 1973; Foteinos et al., 2008; Taylor and Lewis, 1986), this distribution of EC turnover rates may also be caused by the intrinsic differences of proliferative potential among vascular EC (Caplan and Schwartz, 1973; Schwartz and Benditt, 1976, 1977). This suggests that unlike the earlier assumption that all EC possess a similar potential to proliferative, a hierarchy of proliferative potential may exist in the blood vessel EC (Wright, 1972). This was supported by EC colony forming experiments through the *in vitro* culture of isolated resident vascular EC (Alphonse et al., 2014; Alphonse et al., 2015; Alvarez et al., 2008; Huang et al., 2010; Ingram et al., 2005; Nishimura et al., 2015; Schniedermann et al., 2010).

Proliferative endothelial cells

Although, over 4 decades ago, tritiated thymidine labeling experiments discovered that EC in blood vessels have different proliferation rates (Schwartz and Benditt, 1976, 1977), EC are still commonly believed to be identical cells that line the inside of the blood vessels and only proliferate during angiogenesis at a similar rate. However, recently, it was found that though majority of vascular EC are senescent mature cells, some immature EC reside in the vascular beds of murine (Hashimoto et al., 2007; Naito et al., 2012; Naito et al., 2016; Nishimura et al., 2015; Patel et al., 2017; Schniedermann et al., 2010; Wakabayashi et al., 2013), rat (Alphonse et al., 2014; Alphonse et al., 2015; Alvarez et al., 2008), bovine (Huang et al., 2010), monkey (Shelley et al., 2012), porcine (Huang et al., 2007), as well as human (Alphonse et al., 2014; Alphonse et al., 2015; Basile and Yoder, 2014; Green et al., 2017; Ingram et al.,

2005; Rapp et al., 2012) tissues can form EC colonies *in vitro* upon re-plating. These cells were thus named endothelial colony forming cells (ECFC) (Ingram et al., 2004). Additionally, clonal analysis of cultured human umbilical arterial and venous ECFC revealed the existence of a hierarchy of proliferative potential that some immature EC progenitors have the ability to form secondary EC colonies of various sizes, from 2-50 cells to over 10, 000 cells (Green et al., 2017; Ingram et al., 2005). Similar hierarchy of proliferative potential has also been discovered in EC derived from vasculatures of other human tissues (Alphonse et al., 2014; Alphonse et al., 2015; Rapp et al., 2012) and other species (Alphonse et al., 2014; Alphonse et al., 2015; Huang et al., 2010). These experiments demonstrate that just like long term hematopoietic stem cells (HSC) can self-renew and give rise to a hierarchy of short term HSC, hematopoietic progenitor cells (HPC) and mature hematopoietic cells, a similar hierarchy of EC stem / progenitor cells exists in each vascular bed and may contribute to vessel growth during development and tissue regeneration. Indeed, some cardiovascular diseases, like peripheral artery disease (PAD), have been linked with impaired vascular EC colony forming potential (Prasain et al., 2014) while some others, like pulmonary artery hypertension (PAH), were found to be correlated with increased EC colony forming potential (Duong et al., 2011). Thus, identifying EC stem / progenitor populations in the blood vessels is crucial for understanding the mechanism of vessel growth and might be the key for the treatment of various cardiovascular diseases (CVD). Additionally, knowledge about the development, differentiation and maintenance of EC stem / progenitor cells may help researchers to develop more advanced culture methods to efficiently differentiate EC from human

pluripotent stem cells that can be applied to cell therapy for treating CVD (Prasain et al., 2014).

Methods to distinguish vascular endothelial stem / progenitor cells

Despite their importance, the study of vascular endothelial stem / progenitor cells is currently hindered by the lack of proper methods to isolate these cells from other mature EC. Most ECFC methods of identification rely solely on the colony forming function, but lack distinctive surface markers of EC stem / progenitor cells. While most mature EC cannot form secondary colonies and thus can be depleted from the culture, high proliferative EC will give rise to a new hierarchy of EC upon re-plating (Alphonse et al., 2014; Alphonse et al., 2015; Ingram et al., 2005; Rapp et al., 2012) and thus are still indistinguishable from low-proliferative EC in the culture. Some researchers use the side population method, which relies on the ability of some immature EC to efflux the DNA dye Hoechst 33342, to distinguish EC stem / progenitor cells (Naito et al., 2012; Naito et al., 2016; Wakabayashi et al., 2013). As stem / progenitor cells from various lineages, include hematopoietic(Brunet de la Grange et al., 2013; Eaker et al., 2004; Rossi et al., 2011), intestinal epithelial(Dekaney et al., 2005; von Furstenberg et al., 2014), cardiomyocyte (Oyama et al., 2007; Unno et al., 2012) and cancer cells (Christgen et al., 2012; Yasuda et al., 2013) can be isolated using this method, EC with colony forming potential are also highly enriched in side population (SP) cells (Naito et al., 2012; Naito et al., 2016; Wakabayashi et al., 2013). However, since this method is also based on cell function but not surface markers, it is not suitable for the identification of EC stem / progenitor cells *in vivo*

and thus not applicable for tracing the contribution of these cells to the growth of the vasculatures. Recently, some groups have started to use specific cell surface markers, or combination of surface markers, to identify EC stem / progenitor cells. For example, Ralf Adams group identified Endomucin^{+}CD31^{hi} EC population in developing bones that can contribute to the bone vascularization during growth and these cells are crucial for the osteogenesis process (Kusumbe et al., 2014). Patel *et al*. reported that the expression of the surface marker CD34 can predict the high proliferative potential in cultured human ECFC (Patel et al., 2016). The same group reported recently that CD31$^{-/lo}$VEGFR2$^{lo/intracellular}$ EC in mice represent endovascular progenitors that have *in vitro* colony forming potential and *in vivo* vessel forming ability (Patel et al., 2017). Malinverno *et al*. showed that Peg3/PW1 marks murine EC progenitors with superior colony forming potential than PW1^{neg} EC (Malinverno et al., 2017).

All those abovementioned studies, and those colony forming assay-based studies (Alphonse et al., 2014; Alphonse et al., 2015; Alvarez et al., 2008; Basile and Yoder, 2014; Green et al., 2017; Hashimoto et al., 2007; Huang et al., 2010; Ingram et al., 2005; Naito et al., 2012; Naito et al., 2016; Nishimura et al., 2015; Patel et al., 2017; Rapp et al., 2012; Schniedermann et al., 2010; Wakabayashi et al., 2013), focused mainly on the proliferative potential of EC progenitors, especially their *in vitro* EC colony forming ability, but did not directly test whether these EC express true stem properties. The notion of vascular endothelial stem cell (VESC) has only recently been proposed, and only a few studies have targeted VESC identification. Using a SCL-PLAP

reporter mouse line, Canete *et al*. identified that SCL^{+}Vecad (CD144)$^{+}$CD45^{-} EC in the fetal liver are enriched with EC progenitors with long term repopulation potential after transplantation (Canete et al., 2017). Though they did not use the term "VESC", these cells did show long term, multipotent repopulating potential that contributed to liver sinusoidal EC as well as capillary EC in lung, heart and kidney (Canete et al., 2017). Fang *et al*. reported that a single c-kit^{+}CD31^{+}CD105^{+}Scal1^{+}lin^{-} cell derived EC could form functional blood vessels after transplantation into recipient mice (Fang et al., 2012). Yu *et al*. found that protein C receptor (Procr) expressing EC are enriched with VESC that could form *in vitro* EC colonies and contribute to the development of mammary gland, skin and retina blood vessel development (Yu et al., 2016). Additionally, the depletion of Procr^{+} VESC in the neonatal mice could impair the vessel development and regeneration (Yu et al., 2016). To date, all reports that studied VESC were conducted in mice and whether these markers can be applied to human VESC is not known.

The criteria of VESC

While the proliferative potential of EC in the vasculature or circulation has been shown to relate with various CVD (Alvarado-Moreno et al., 2016; Lee et al., 2015; Prasain et al., 2014), the theory of VESC is yet to be appreciated by the broad endothelial biology field. The definition of VESC is still vague and needs to be clarified. Lineage specific stem cells should display potential for clonal proliferation and contribute to all cell types in this lineage and, importantly, have the ability to self-renew and thus provide long-term support to tissues. Therefore, a true VESC should

fulfill the following criteria: a), Ability to form *in vitro* EC colonies upon re-plating. b), Capacity to form functional blood vessels *in vivo* after transplantation into recipient animals. c), Potential to different into arterial EC, venous EC and capillary EC *in vivo*. d), Self-renewal ability. (Figure I. 2)

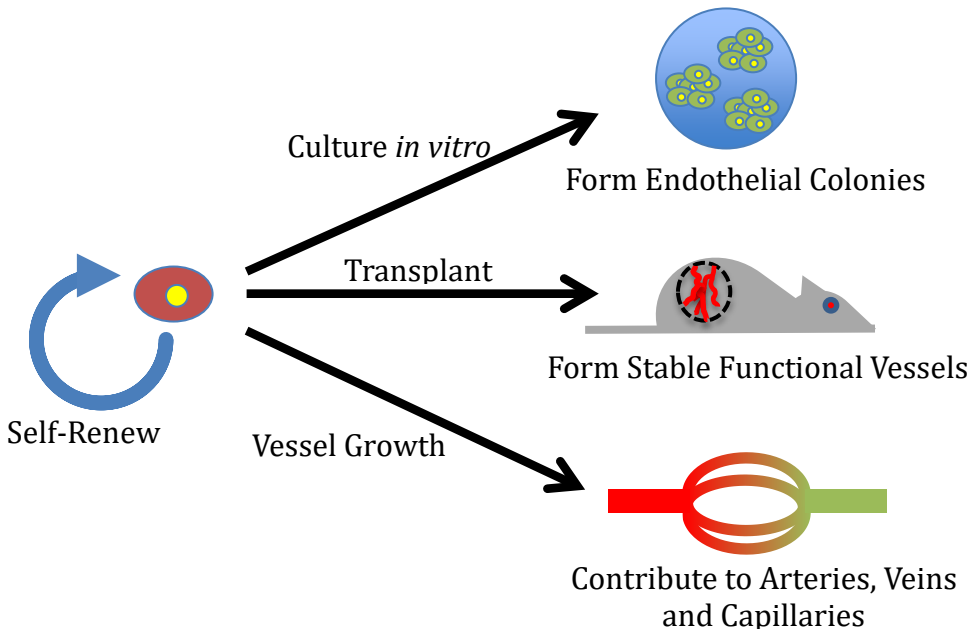


Figure I. 2 Criteria of vascular endothelial stem cells.

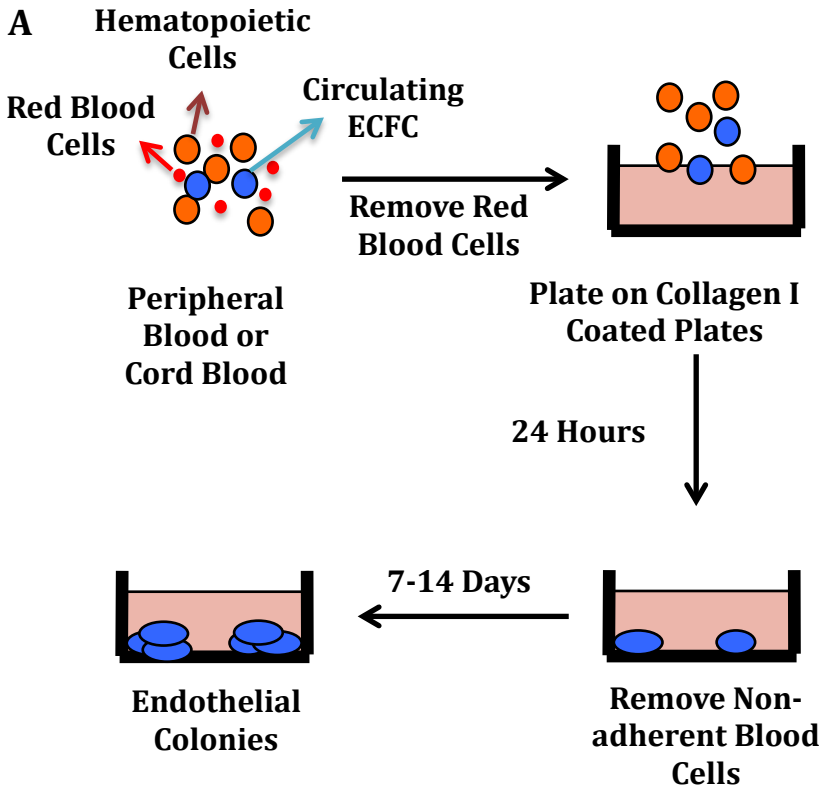
3. Circulating endothelial colony forming cells (ECFC)

Pro-angiogenic hematopoietic cells and circulating ECFC

The existence of circulating cells that can form endothelial colonies on the surface of blood vessel implants in experimental animals has been known for over five decades (Stump et al., 1963). However the searching for the origin of endothelial lineage circulating progenitors is still ongoing. In 1997, Asahara's group announced the identification of circulating cells that exhibited endothelial phenotypes like LDL uptake and EC morphology *in vitro* (Asahara et al., 1997). Later, more circulating cell types with similar features were identified by various groups through morphology, cell surface marker expression, LDL uptake, or the ability to promote vessel growth (Asahara et al., 2011; Asahara et al., 1999; Hill et al., 2003; Peichev et al., 2000; Vasa et al., 2001). Such cells were given the term "endothelial progenitor cells (EPC)" for their EC-like properties (Asahara et al., 1997). In addition, several methods to identify putative EPC through *in vitro* culture were developed including CFU-Hill (Asahara et al., 1997; Hill et al., 2003), circulating angiogenic cells (CAC) (Kalka et al., 2000), and the ECFC method (Ingram et al., 2004; Javed et al., 2008). However, most of early studies on EPC lacked rigorous characterization of EC functions and thus the term "EPC" actually refered to a mixed population of myeloid lineage hematopoietic cells and EC (Rehman et al., 2003; Yoder, 2013; Yoder et al., 2007). Indeed, though the EC-like cells identified using CFU-Hill or CAC methods show up earlier (3-9 days after culture), exhibit many EC features and promote the formation of blood vessels *in vivo* (Asahara et al., 1997; Hill et al., 2003; Vasa et al., 2001), they lack the potential to

participate in blood vessels as a long-term structural component (Rehman et al., 2003; Shinde Patil et al., 2005; Yoder, 2013; Yoder et al., 2007). Instead, their pro-angiogenic effects are mostly conducted through paracrine effects (Medina et al., 2017; Yoder, 2013). Therefore, "EPC" is no longer an accurate term to define such cells and is gradually being replaced by "pro-angiogenic hematopoietic cells" (Kopp et al., 2006; Medina et al., 2017; Rose et al., 2015; Yoder, 2013). In addition, more stringent EC function assays, like the 3D collagen tube forming assay (Critser and Yoder, 2010) and *in vivo* vessel forming assays, are required for the identification of true endothelial lineage progenitor cells. Only cells derived from the ECFC culture method, which emerge 10-14 days after plating and thus are also called "late outgrowth endothelial cells" or "blood outgrowth endothelial cells" (Hirschi et al., 2008; Ingram et al., 2004; Javed et al., 2008), represent authentic EC progenitor populations that have the ability to form secondary EC colonies upon re-plating and form stable, functional blood vessels after transplantation (Hirschi et al., 2008; Ingram et al., 2004; Javed et al., 2008; Melero-Martin et al., 2007; Schechner et al., 2000) (Figure I. 3). Circulating ECFC derived from human cord blood (CB) or peripheral blood (PB) showed robust colony proliferative potential. After re-plating, some single cells from circulating ECFC derived cells can form EC colonies that contain more than 10, 000 cells (Ingram et al., 2004; Javed et al., 2008; Prasain et al., 2014). In addition, after implanted into host animals, circulating ECFC derived cells have robust potential to form functional blood vessels that are inosculated with host blood vessel system (Ingram et al., 2004; Javed et al., 2008; Prasain et al., 2014) (Figure I. 4). Furthermore, compared to vascular resident EC, circulating ECFC are

easy to access from patient CB and PB mononuclear cells (MNC), and more circulating ECFC can be mobilized into the patients' circulating via the administration of mobilizers like AMD3100 or G-CSF (Shepherd et al., 2006b).


A
Hematopoietic Cells
Red Blood Cells
Circulating ECFC
Remove Red Blood Cells
Peripheral Blood or Cord Blood
Plate on Collagen I Coated Plates
24 Hours
7-14 Days
Endothelial Colonies
Remove Non-adherent Blood Cells

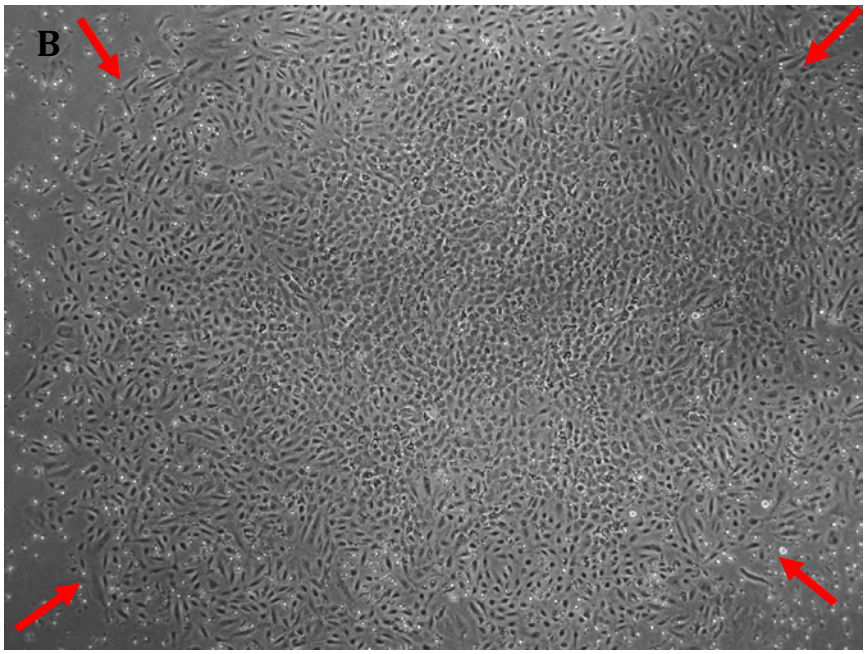

B

Figure I. 3 The isolation of circulating endothelial colony forming cells (ECFC). (A) Method to isolate circulating ECFC from human peripheral blood or cord blood. (B) A representative picture of human cord blood derived circulating ECFC colony after 10 days of culture.

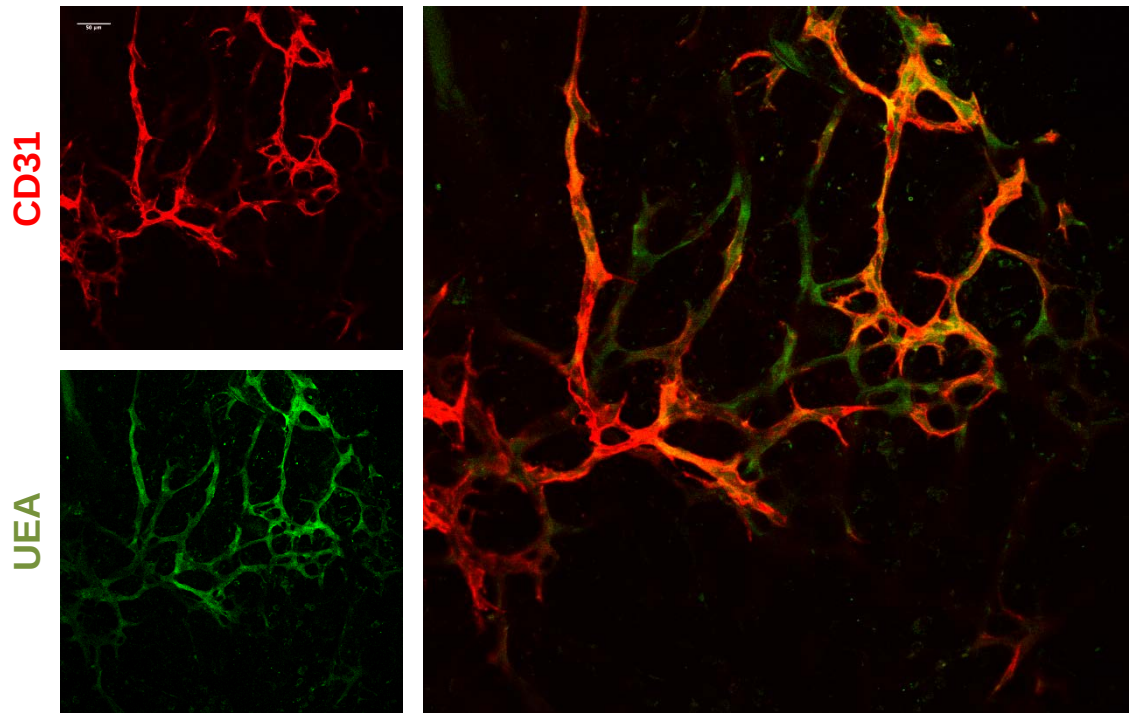


Figure I. 4 Human endothelial colony forming cell (ECFC) derived blood vessels after transplantation. Human ECFC formed robust blood vessels that expressed human CD31 after transplantation into immunodeficient mice. The perfusion of these vessels were shown by intravenous injecting and labeling by the human specific lectin Ulex Europaeus Agglutinin I (UEA 1).

Therapeutic potential of circulating ECFC

These features of circulating ECFC render them very promising candidate for the treatment of various CVD, especially those characterized by compromised EC proliferation potential, like PAD (Brevetti et al., 2008; Hirsch et al., 2001; Prasain et al., 2014). Indeed, the change of frequency, proliferative potential and function of circulating ECFC has been linked with many CVD including PAD (Mund et al., 2012), venous thromboembolic disease (Alvarado-Moreno et al., 2016), pediatric Moyamoya Disease (Lee et al., 2015), coronary artery disease (Chang et al., 2017), bronchopulmonary dysplasia (Alphonse et al., 2014; Baker et al., 2012; Borghesi et al., 2009), and myocardial infarction (Huang et al., 2007). The administration of circulating ECFC, or pluripotent stem cell derived circulating ECFC-like cells into model animals that mimic various CVD phenotype, has shown very promising therapeutic benefits (Alphonse et al., 2014; Huang et al., 2013; Hur et al., 2004; Medina et al., 2010; Moubarik et al., 2011; Prasain et al., 2014; Zhang et al., 2013). In addition, unlike pro-angiogenic hematopoietic cells or mesenchymal lineage cells, which improve blood perfusion via indirect mechanisms and do not long-term incorporate into the blood vessels (Bronckaers et al., 2014; Huang and Li, 2008; Medina et al., 2017; Yoder, 2013), circulating ECFC can also incorporate long-term into newly formed blood vessels as stable structural elements (Ingram et al., 2004; Javed et al., 2008; Medina et al., 2017; Melero-Martin et al., 2007; Yoder et al., 2007). Thus, circulating ECFC derived EC can provide long-term support to the vasculature in hosts, which is especially important for the treatment of CVD patients with impaired EC proliferative potential, like PAD patients (Brevetti et al., 2008; Hirsch et

al., 2001; Prasain et al., 2014). Circulating ECFC may also be a promising source of EC for engineering of vascularized tissue grafts (Kung et al., 2008; Kuo et al., 2015; Shepherd et al., 2006a) (Critser and Yoder, 2010).

Challenges for circulating ECFC study

Despite the therapeutic potential of circulating ECFC, many fundamental biological questions remain open. For example, though circulating ECFC show robust proliferative potential and blood vessel forming ability in *in vitro* and *in vivo* tests, their biological functions during vessel development and tissue regeneration are still not clear. It is known that compared to resident EC, human circulating ECFC highly express surface adhesion molecules like P-selectin glycoprotein ligand-1 (PSGL-1) (Foubert et al., 2007; Hubert et al., 2014), a ligand for E- and L- Selectin, which are expressed by neutrophils (Abbassi et al., 1993; Finger et al., 1996). In addition, CXCR4, the receptor for chemokine CXCL12 (SDF1-α), is also expressed by human circulating ECFC (Newey et al., 2014; Smadja et al., 2014; Sun et al., 2013). The CXCR4-CXCL12 axis is an important mechanism for the homing of hematopoietic stem/progenitor cells to the bone marrow niche (Sugiyama et al., 2006) or to the injury sites (Yellowley, 2013). Thus, a model of the recruitment of circulating ECFC to an injured site has been proposed (Basile and Yoder, 2014; Ingram et al., 2005; Yoder and Ingram, 2009): After injury, pro-angiogenic hematopoietic cells are recruited and adhere to the wound rapidly. Next circulating ECFC are also recruited through the gradient of CXCL12 and adhere to E- and L- Selectin expressing pro-angiogenic hematopoietic cells at the injury sites to initiate the injury healing process. In

accordance to this model, CXCL12 has been shown to be able to enhance the integration of circulating ECFC into the vascular system (Newey et al., 2014), and the antagonist of CXCR4, AMD3100, could disrupt the formation of EC network (Newey et al., 2014) and mobilize circulating ECFC from treated patients (Shepherd et al., 2006b). Additionally, using intravital microscopy, the process of neutrophil recruiting circulating ECFC after vessel injury via L-Selectin – PSGL1 interaction has been monitored (Hubert et al., 2014). Thus, the most likely *in vivo* function of circulating ECFC is repairing damaged blood vessels, though more direct evidence is still needed.

In addition, lineage specific stem cells, like hematopoietic stem cells (Giebel and Bruns, 2008; Seita and Weissman, 2010) and neural stem cells (Doe, 2008; Molofsky et al., 2003), can produce progenitor cells with proliferative potential to give rise to a large number of progeny, and also have the ability to self-renew. It is still not known whether circulating ECFC are just progenitor cells that can only divide for a limited number of times, or this cell population also contains endothelial stem cells with self-renew ability. *In vitro* cultured human circulating ECFC derived cells can form a hierarchy of single cell derived EC colonies with different sizes (Ingram et al., 2004; Javed et al., 2008). Once cells from those colonies were re-plated at a single cell level, only some EC from those largest colonies (contain more than 10, 000 EC) have the potential to give rise to secondary EC colonies that recapitulate the same hierarchy, while cells from smaller colonies can only grow into colonies that have similar or smaller size than the primary colonies, (Ingram et al., 2004; Javed et al., 2008). That

suggests at least some cells in circulating ECFC derived cells have self-renew potential *in vitro*. However whether these cells can self-renew *in vivo*, which means, not only just produce more EC to integrate into the blood vessels, but also display capacity to regenerate high-proliferative potential resident ECFC that can provide long-term support to the blood vessels, is not known. Furthermore, a lineage specific stem cell must display the potential to differentiate into all cell types in this lineage (Doe, 2008; Giebel and Bruns, 2008; Molofsky et al., 2003; Seita and Weissman, 2010), for the endothelial lineage, if circulating ECFC contain circulating endothelial stem cells, they should have the potential to differentiate into arterial, venous and capillary EC. It has been reported that circulating ECFC derived EC, while mainly form capillaries after transplantation in collagen gel plugs, but can differentiate into arterial EC when co-transplanted with NOTCH ligand DL1-overexpressing OP9 stromal cells (Ingram et al., 2004; Javed et al., 2008; Kim et al., 2015; Prasain et al., 2014). However more studies on this topic at a single cell level is required to determine the full lineage regeneration potential of circulating ECFC.

The origin of circulating ECFC is another controversial question. Circulating ECFC isolated from human patients receiving sex-mismatched bone marrow transplant were shown to be donor derived, while low-proliferative potential circulating EC were all originated from the host (Lin et al., 2000). However, viable circulating EC in the quail embryo that could contribute to vessel development were reported to be derived from the vasculature (Cui et al., 2013), and resident ECFC with comparable proliferative potential as CB circulating ECFC have been isolated from the vessel walls

of umbilical arteries and veins (Ingram et al., 2005). Thus, a vascular origin of circulating ECFC has also been suggested (Yoder, 2010).

The existence of those challenges for the study of circulating ECFC is partially due to the lack of a specific combination of cell surface markers to distinguish circulating ECFC from mature EC in the circulation. It is known that cultured circulating ECFC derived cells express EC markers like CD31, KDR, VE-Cadherin, and CD34, but not hematopoietic-specific markers like CD45, CD115 or CD11b (Basile and Yoder, 2014; Ingram et al., 2004; Medina et al., 2013; Tasev et al., 2016). However, due to their scarce distribution in human PB or CB (Ingram et al., 2004; Javed et al., 2008), circulating ECFC's *in vivo* markers before re-plating have yet to be defined. Using fluorophore conjugated monoclonal antibodies, magnetic activated cell sorting (MACS) and flow cytometry analysis combined with *in vitro* culture, human CB circulating ECFC are enriched in the $CD31^{+}CD146^{+}CD34^{+}CD45^{-}AC133^{-}$ cell fraction (Case et al., 2007; Mund et al., 2012). 4 in 10, 000 $CD34^{+}CD45^{-}$ cells isolated from human CB could form endothelial colonies while CB $CD34^{+}AC13^{+}KDR^{+}$ cell population, once believed to be the markers for human circulating "EPC" (Asahara et al., 2011; Asahara et al., 1999; Asahara et al., 1997; Hill et al., 2003), did not show any EC colony forming potential (Case et al., 2007). However, though these markers can be used to greatly enrich the cell population that contain circulating ECFC *in vivo*, circulating ECFC are rare in human CB and PB; the average number of circulating ECFC in CB MNC is 1 in 10^{6} cells (Case et al., 2007), or 8 circulating ECFC in 20ml human CB (Ingram et al., 2004), or 0.5 circulating ECFC in 20ml adult human PB

(Ingram et al., 2004)), specifically isolating circulating ECFC from blood MNC is still a challenge. To better understand the mechanism of circulating ECFC emergence, function and their relationship with various CVD, more studies on circulating ECFC isolation and detection are required.

Another impediment on research on circulating ECFC is the lack of appropriate mouse models. Though circulating ECFC have been identified from various species samples including human (Ingram et al., 2005; Ingram et al., 2004; Javed et al., 2008), rhesus monkey (Shelley et al., 2012), porcine (Huang et al., 2007), and bovine (Critser et al., 2011) blood, they were believed not to exist in the circulation of each individual healthy mice (Somani et al., 2007). Indeed, using the same methods to culture human circulating ECFC, only 1 circulating ECFC could be detected from blood isolated from over 50 mice that were older than 8 weeks and 1 from 20 mice that were younger than 8 weeks (Somani et al., 2007). In human subjects, umbilical CB contains more circulating ECFC with greater proliferative potential and blood vessel forming potential than adult peripheral blood (Au et al., 2008; Ingram et al., 2004; Javed et al., 2008; Melero-Martin et al., 2008). In the rhesus monkey, both the number and the *in vivo* blood vessel forming potential of circulating ECFC declines with age (Shelley et al., 2012). Thus, to find circulating ECFC in mouse blood, embryonic / neonatal / juvenile PB might be more promising than adult PB. Co-culture murine EC with OP9, a type of murine stromal cell derived from stroma of the mouse calvarium, is known to be an effective way to culture murine embryonic stem cell derived EC (Hirashima et al., 1999), adult EC from various tissues (Naito et al., 2012; Naito et al., 2016), or embryonic

EC (Hashimoto et al., 2007). Therefore, this method may promote the culture of murine circulating ECFC.

Chapter II

ABCG2 Marks Murine and Human Vascular Endothelial Stem Cells

1. Introduction

All mammals possess a blood vascular system lined with EC that provide a dynamic interface between blood and surrounding tissues, regulate nutrient, waste, and blood cell traffic, and participate in regulating hemostasis, inflammation, and angiogenesis. While thousands of articles have been published on angiogenic growth mechanisms, to date, the specific cellular mechanisms for the replacement of damaged, diseased, or senescent vascular EC in intact blood vessels is unclear. It is well known that cells from many tissue lineages, like hematopoietic cells and intestine epithelial cells, are maintained by lineage-specific stem cells that can self-renew and differentiate into mature progeny (Barker, 2014; Eaves, 2015; Lin et al., 2014), but the existence of endothelial stem cells is less widely accepted. Some EC that can give rise to robust *in vitro* EC colonies and display vasculogenic properties have been identified from mammalian blood vessels (Alphonse et al., 2015; Ingram et al., 2005; Naito et al., 2012; Naito et al., 2016; Nishimura et al., 2015; Patel et al., 2017) or from circulating blood (Ingram et al., 2004; Javed et al., 2008; Shelley et al., 2012). We and others have used the ability of cells to efflux the DNA dye Hoechst 33342 (such cells are called the side population) to isolate quiescent EC with clonogenic and vasculogenic stem cell-like properties, but this phenotype is based on function rather than a cell surface marker and is therefore not feasible to use to prospectively identity these cells *in vivo* (Alphonse et al., 2015; Ingram et al., 2005; Naito et al., 2012; Naito et al., 2016; Patel

et al., 2017). Recently, several groups have reported the identification of quiescent EC possessing proliferative potential in different developmental stages of murine blood vessel development via the differential expression of specific cell surface markers (Canete et al., 2017; Fang et al., 2012; Kusumbe et al., 2014; Malinverno et al., 2017; Patel et al., 2017; Yu et al., 2016). However, whether these EC fulfill all the criteria of unipotent vascular endothelial stem cells (VESC) including clonal proliferative potential, ability to self-renew, contribution to multiple blood vessel compartments (artery, vein, capillary) upon transplantation, and long term contributions to vessel compartments via fate mapping analysis, has not been thoroughly tested. In addition, no putative murine VESC markers have been validated as stem cell markers for human blood vascular EC.

ATP binding cassette family drug transporter ABCG2 is expressed by various lineage-specific stem cells in different tissues (Doyle et al., 2011; Fatima et al., 2012; Maher et al., 2014; Tadjali et al., 2006; Zhou et al., 2001). Additionally, *Abcg2*-expressing EC have been shown to contribute to vessel regeneration after vascular injuries (Doyle et al., 2011; Maher et al., 2014). In this study, we tested the hypothesis that in both human and mice, *ABCG2* (*Abcg2*) - expressing EC represent VESC in the vasculatures. We showed that VESC that express *ABCG2* (*Abcg2*) are distributed in arteries, veins and capillaries. These cells possess *in vitro* colony forming potential, *in vivo* vessel forming ability, the capacity to self-renew *in vivo*, and can contribute to the growth of vasculatures during development and injury healing process.

2. Materials and methods

Animals

All animal experiments were conveyed in accordance with the Guidelines for the Care and Use of Laboratory Animals, and all protocols were approved by Institutional Animal Care and Use Committee of the Indiana University School of Medicine. C57BL/6 (JAX stock #000664), FVB/NJ (FVB, JAX stock #001800), B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J(Madisen et al., 2010) (R26R-TdTomato, JAX stock #007914), NU/J (athymic nude, JAX stock #002019), NOD.CB17-Prkdc^{scid}/J (NOD/SCID, JAX stock #001303) were purchased from the Jackson Laboratory. Bcrp constitutive knock out (ABCG2 knockout, #2767) were obtained from Taconic. CD1 mice (#022) were purchased from Charles River Laboratories. Sperm of ABCG2CreERT mice (Fatima et al., 2012) were kindly provided by Dr. Sorenttino and the colony was recovered in the Indiana University School of Medicine Laboratory Animal Resource Center. The following primers were used for genotyping the abovementioned Cre or ROSA mice:

Cre F: 5'-CGG TCG ATG CAA CGA GTG AT-3'

Cre R: 5’- CCA CCG TCA GTA CGT GAG AT -3’

ROSA F: 5’- CTG TTC CTG TAC GGC ATG G -3'

ROSA R: 5’- GGC ATT AAA GCA GCG TAT CC -3'

Drug administration

Tamoxifen (Sigma-Aldrich) was suspended in sunflower seed oil (Sigma-Aldrich) at 37°C to make a 4mg/ml solution and was stored at -20°C until use. To induce Cre

expression in ABCG2CreERT mice, 50mg/kg tamoxifen was injected into the animals intra-peritoneally (i.p.) at appropriate time points. Tetrandrine solution was prepared by dissolving tetrandrine powder (Santa Cruz Biotechnology) in several drops of 0.01N HCl solution and Ph was adjusted to 5.5 using 0.01N NaOH. PBS was then added to adjust the final concentration to 5mg/ml. The solution was freshly prepared prior to each injection. To block ABCB1a/1b transporter function, 50mg/kg tetrandrine was i.p. injected daily into wild type FVB or ABCG2 knockout pups for 4 consecutive days. PBS was injected in littermates as a control.

Cell collection

To collect cells from mouse lung, liver or heart, tissues were dissected from euthanized mice and were minced with razor blades. Then the samples were digested with 0.25% collagenase I (Stem Cell Technologies) at 37°C for 30 minutes. After digestion, the samples were re-suspended in medium, pipetted thoroughly, and passed through 70μm cell strainers to removed cell clumps. To collect mouse bone marrow cells, tibias and femurs were dissected and cleaned with scissors to remove remaining muscle tissues. Then the bones were crushed with a pestle in a mortar before the cells were digested and filtered like the other tissues.

To collect human umbilical cord artery or vein EC, the umbilical vessels were flushed with PBS 3 times. Then one end of the vessel was clamped and liberase solution (Roche, 500μl stock solution diluted with 24.5ml PBS) was infused into the vessel through the open end before it was clamped. The liberase infused tissues were

incubated at 37°C for 14 minutes to release EC from the basement membrane. Finally, the solution containing digested EC was flushed into 50ml tubes for centrifugation and EC recovery.

Magnetic Activated Cell Sorting (MACS)

ABCG2^{+} cells from human umbilical cord vein EC or MNC were sorted using biotin-anti human ABCG2 antibody (eBioscience, clone 5D3) and EasySep™ Biotin Positive Selection Kit (Stemcell technologies). After sorting, the purity of sorted cells, the percentage of CD31^{+}CD45^{-} EC, or CD34^{+}CD45^{-} MNC were measured by flow cytometry (see below).

Flow cytometry

The following anti-mouse antibodies conjugated with different fluorochrome were used for flow cytometry sorting and analysis: CD31 (clone 390), CD45 (30-F11), Ter119 (TER-119), (all above antibodies were purchased from eBioscience). For human cell flow cytometry analysis, the following anti-human antibodies were used: CD31 (BD Pharmingen or eBioscience, clone WM59), CD45 (eBioscience or BioLegend, clone 2D1), ABCG2 (eBioscience, clone 5D3). 1:1000 propidium iodide (PI, Sigma-Aldrich) was added to sorting buffer before analysis to distinguish dead from live cells. Cell analysis and sorting were performed on LSR 4, LSRII, FACSCantoII, FACSAria, and SORPAria flow cytometers (BD Biosciences). FlowJo software was used to analyze flow cytometry data.

For side population staining, cells were stained for surface antigens first and then every 1 million of MNC were re-suspended in 1 ml of SP buffer (DMEM with 2% FBS and 1mM HEPES). Next 5μg/ml Hoechst 33342 (Sigma-Aldrich) was added to the cell suspension and incubated at 37°C for 90 minutes with or without 50μmol/l verapamil (Sigma-Aldrich). Finally the cells were re-suspended in sorting buffer before they were analyzed/sorted with SORPAria flow cytometer with a UV laser.

Culture of Endothelial Colonies

For murine EC culture, OP9 stromal cells were maintained in OP9 medium (alpha-MEM medium [Gibco], with 20% FBS [Hyclone], and 0.5% penicillin/streptomycin [Gibco]). To culture endothelial colonies, isolated murine endothelial cells were re-suspended in EC culture medium (alpha-MEM with 10% FBS [Hyclone], 5×10^{-5} M β-mercaptoethanol [Sigma-Aldrich] and 0.5% penicillin/streptomycin [Gibco]). After 24 hours, non-adherent cells were removed by changing fresh medium. Medium was changed every 3 days afterwards until use. For human EC culture, the cells were re-suspended in complete EGM2 medium (Endothelial Basal Medium -2 [EBM-2, Lonza] with 10% FBS [Hyclone]) and re-plated on 0.1% type 1 rat tail collagen (BD Biosciences) coated tissue culture plates.

3D *in vitro* tube forming assay in collagen gel

200Pa stiffness pig skin type I collagen gels were made according to the manufacturer's instructions (Geniphys, Standarized Oligomer Polymerization Kit). 10% human platelet lysate (Cook) was then added into the gel and the liquid gel was

kept on ice. Human EC were re-suspended in the gel at a density of ~1 × 10^6 cells/1ml gel and each 50µl gel was transferred to a well in 96 well plate. The cellularized gels were incubated at 37°C for 30 minutes to solidify. Next the gels were covered by adding 100µl complete EGM2 medium to the wells. The cultures were checked under a microscope for every 12 hours until lumenized vessel-like structures were formed and identified.

Surgeries

For EC collagen gel transplantation assay, cells were re-suspended in 250ul 200pa pig skin type I collagen collagen gel (Geniphys, Standarized Oligomer Polymerization Kit) plus 10% human platelet lysate (Cook) on ice. When murine EC were tested, 50µg/ml murine VEGF (Peprotech) and 100µg/ml murine FGF (Peprotech) were added to the gels. Each cellularized gels was transferred into 1 well in a 48 well plate and incubated at 37 ˚C to polymerize for 30 minutes. Next the cellularized gels were transplanted subcutaneously into the flanks of 6-12 weeks old NOD/SCID mice as previously described (Prasain et al., 2014). The gels were retrieved from the animals at various time points between 14 days and 10 months past-implant.

Hind limb ischemia experiments were conducted as previously described(Prasain et al., 2014). Briefly, after 6-8 week old athymic nude mice were anesthetized with isoflurane, a skin incision on their left thigh were made. The distal and proximal ends of the femoral artery were ligated and the portion of femoral artery between these two ligation sites were excised. After the excision, 200µl cell suspension in PBS or

control PBS, were injected into 4 sites of gracilis muscle. Then the incisions were sutured closed and a Laser Doppler imager (Moor Instruments) was used to measure the blood flow in the injured and control legs on days 0 and every 7 days post-treatment until 6 weeks. The mean profusion values from each leg was analyzed and calculated using Moor software (Prasain et al., 2014).

Cell culture immunohistochemistry and immunofluorescent staining

For immunohistochemistry staining of endothelial colonies on OP9 co-culture plates, the cultures were fixed with 4% paraformaldehyde (PFA) for 30 minutes at RT. After washing, the samples were blocked with 2% skim milk (Sigma-Aldrich) in 0.1% triton (Sigma-Aldrich) PBS solution (PBSMT solution) for 30 minutes at RT and then stained with 1:100 rat anti mouse CD31 (BD Pharmingen, clone MEC 13.3) or rat anti mouse Flk1 (BD Pharmingen, clone Avas 12α1) antibody in PBSMT at RT for 2 hours or at 4°C overnight. Next the plates were stained with 1:200 alkaline phosphatase conjugated donkey anti rat IgG secondary antibody (Jackson ImmunoResearch) in PBSMT at RT for 2 hours or at 4°C overnight. The colonies were visualized by VECTOR Blue Alkaline Phosphatase (Blue AP) Substrate Kit.

For culture immunofluorescent staining of murine EC culture, fixed cultures were blocked with 10% goat serum in 0.5% triton PBS solution (blocking solution) and sequentially stained with primary antibody (1:100 rat anti mouse CD31, clone MEC 13.3) and secondary antibody (1:200 Alexa Fluor 488 or 647 conjugated goat anti rat IgG [Cell Signaling Technology]) in blocking buffer. All culture pictures were visualized using Leica™ DM IL microscope with a SPOT RT3 camera (Spot Imaging).

Tissue immunofluorescent staining

To visualize Tdtomato+ vessels in freshly collected muscle or collagen gel samples after transplantation, a LeicaTM mz9.5 stereomicroscope with LEJ eqb 100 isolated lamp power supply was used. To take confocal images of tissues or transplanted gels, the samples were collected and fixed in 4% PFA at 4°C overnight, rinsed in 30% sucrose o at 4°C overnight, and then mounted in O.C.T. compound (Fisher Scientific) on dry ice. The tissue blocks were cut into 10-30μm sections using a Leica CM3050s cryostat and mounted on Superfrost Plus Gold microscope glass slides (Thermo Fisher Scientific). After blocking with blocking buffer at RT for 1 hour, the slides were then stained with different unconjugated primary anti bodies include: rat anti mouse CD31 (BD Pharmingen, clone MEC 13.3, 1:100), rabbit anti ERG (Abcam, clone EPR3864. 1:100), or mouse anti human ABCG2 (Abcam, clone BXP-21. 1:50), at 4°C overnight. Then 1:200 Alexa Fluor 488, 555, or 647 conjugated goat anti-rat, anti-rabbit, or anti-mouse IgG antibodies (Cell Signaling Technology) were used for secondary staining at 4°C overnight. For some stainings, the following conjugated antibodies were used: Alexa Fluor 647 conjugated mouse anti human CD31 (BD Pharmingen, Clone WM59. 1:50), Alexa Fluor 488 or 594 conjugated mouse anti smooth muscle actin α (eBioscience, clone 1A4. 1:100). After staining, the samples were mounted with ProLong™ Gold Antifade Mountant with DAPI (Molecular Probes) and Z-stack confocal images were taken on Olympus FV1000 microscope.

All fluorescent pictures were processed using ImageJ software to produce merge images. 3D reconstruction of $CD31^+$ and $TdTomato^+$ vessels in tissues or gels were

performed using Imaris software. The volumes of blood vessels were calculated by Imaris software using "Surface" function according to the manufacturer's instruction.

Quantitative PCR

RNA from each sample was extracted using RNeasy Micro kit (Qiagen). Reverse transcription was done using Omniscript RT Kit (Qiagen). Quantitative PCR was performed on Applied Biosystems® 7500 Real-Time PCR System with FastStart Universal SYBR Green Master (Roach) in triplicate. Beta-actin was used as reference gene to calculate transcript abundance of each target gene. The expression level fold change between sample genes and reference genes were calculated by 7500 software. The following primers were used:

ABCG2F: 5'-CCATAGCCACAGGCCAAAGT-3'

ABCG2R: 5'-GGGCCACATGATTCTTCCAC-3' (ref The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype)

ABCB1aF: 5'-CCAGCAGTCAGTGTGCTTACA-3'

ABCB1aR: 5'-GCCACTCCATGGATAATAGCA-3'

ABCB1bF: 5'-TGATCATCAGCAACAGCAGTC-3'

ABCB1bR: 5'-TGAAACCTGGATGTAGGCAAC-3'

ABCB2F: 5'-CTCTTGCCTTGGGGAAATG-3'

ABCB2R: 5'-CTGTGCTGGCTATGGTGAGA-3'

ABCC7F: 5'-GACACTTTGCTTGCCCTGAG-3'

ABCC7R: 5'-AAGAATCCCACCTGCTTTCA-3'

ABCA5F: 5'-TTCTATGTCCTCCTGGCTGTG-3'

ABCA5R: 5'-TGACCAATACGATGGCTTCA-3'

ABCA3F: 5'-TTATGCCCTCCTACTGGTGTG-3'

ABCA3R: 5'-CTTGTCCTTATTGCCCACTTG-3' (abovementioned primers from (Naito et al., 2016))

ABCG2F: 5'-CCATAGCCACAGGCCAAAGT-3'

ABCG2R: 5'-GGGCCACATGATTCTTCCAC-3' (above two primers from (Zhou et al., 2001))

Beta-actinF: 5'- TCCTGTGGCATCCATGAAACT-3'

Beta-actinR: 5'- GAAGCACTTGCGGTGCACGAT-3'

Western blot analysis

Protein from each kidney was extracted by mincing in an ice cold mortar and re-suspended in 150µl lysis buffer (0.5M HEPES, 5M Nacl, 50% Glycerol, 25%)

Cells were washed twice with ice-cold phosphate-buffered saline and lysed on ice in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor (Roche). Cell lysates were sonicated and centrifuged at 13,200 rpm for 10 min; boiled with LDS sample buffer (ThermoFisher Scientific); separated by NuPAGE gel (ThermoFisher Scientific); transferred electrophoretically to a PVDF (EMD Millipore); and immunoblotted with Abcg2 antibody (Abcam, clone BXP-21), and Gapdh antibody (Cell Signaling Technology), followed by incubation with HRP-conjugated secondary antibodies (Cell Signaling Technology). The blots were developed using the enhanced

chemiluminescence technique with HRP substrate peroxide Solution (EMD Millipore).

Statistical analyses

Unless otherwise mentioned, all data are presented as mean ± standard deviation and unpaired two tailed Student's t-test was used to determine significance. If p value > 0.05, non-significant (n.s.) was considered. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. All statistical analysis were implemented using Graphpad Prism or Microsoft Excel software. n represent the number of mice or number of human patients that were used in each experiment and the numbers were provided in figure legend for each experiment.

3. Results

Abcg2 is critical for the maintenance of vascular endothelial colony forming cells

Vascular beds from multiple murine tissues contain rare progenitor cells that can form EC colonies *in vitro* (Figure II. 1). Since the side population (SP) phenotype (Figure II. 2A), labels stem/progenitor cells in multiple lineages (Challen and Little, 2006) including some quiescent vascular EC (Naito et al., 2012; Naito et al., 2016; Patel et al., 2017) (Figure II. 2B), we examined the level of transcript expression of ABC family members known to be important for drug efflux, in the endothelial SP in comparison to the main population (do not possess high Hoechst 33342 efflux function; MP). The primary murine lung EC SP fraction highly expresses *Abcg2 (Bcrp2)* mRNA, as well as, two other members of the ABC family of transporters, *Abcb1a (Mdr1a)* and *Abcb1b (Mdr1b)* (Figure II. 3). The lung SP fraction was also significantly enriched in endothelial colony forming cells (ECFC) (Figure II. 2B).

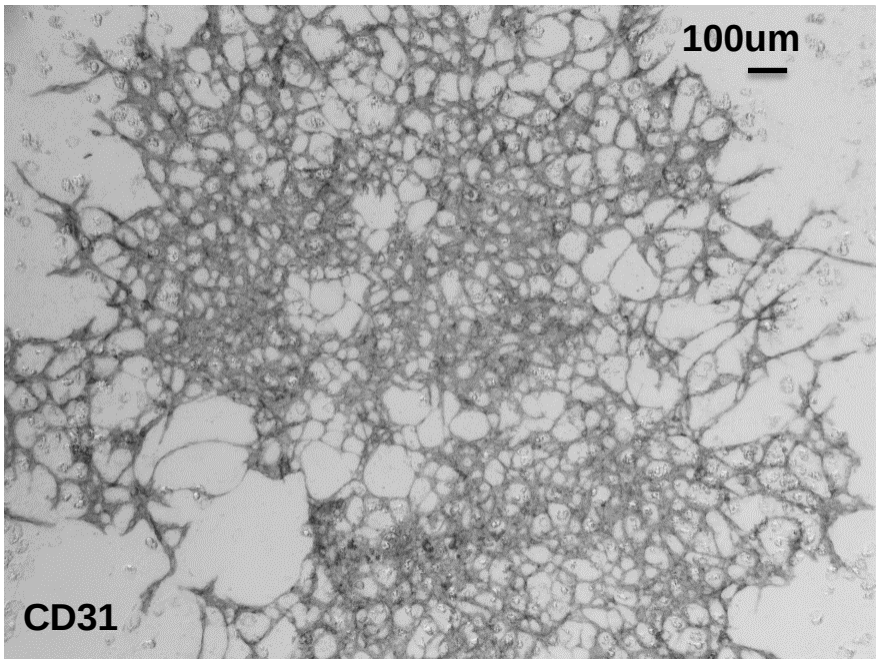


Figure II. 1. Representative picture of an alkaline phosphatase stained $CD31^+$ EC colony derived from lung $CD45^-CD31^+$ EC in OP9 co-culture. The colony was visualized by alkaline phosphatase after stained by rat anti-mouse CD31 and alkaline phosphatase conjugated anti-rat IgG.

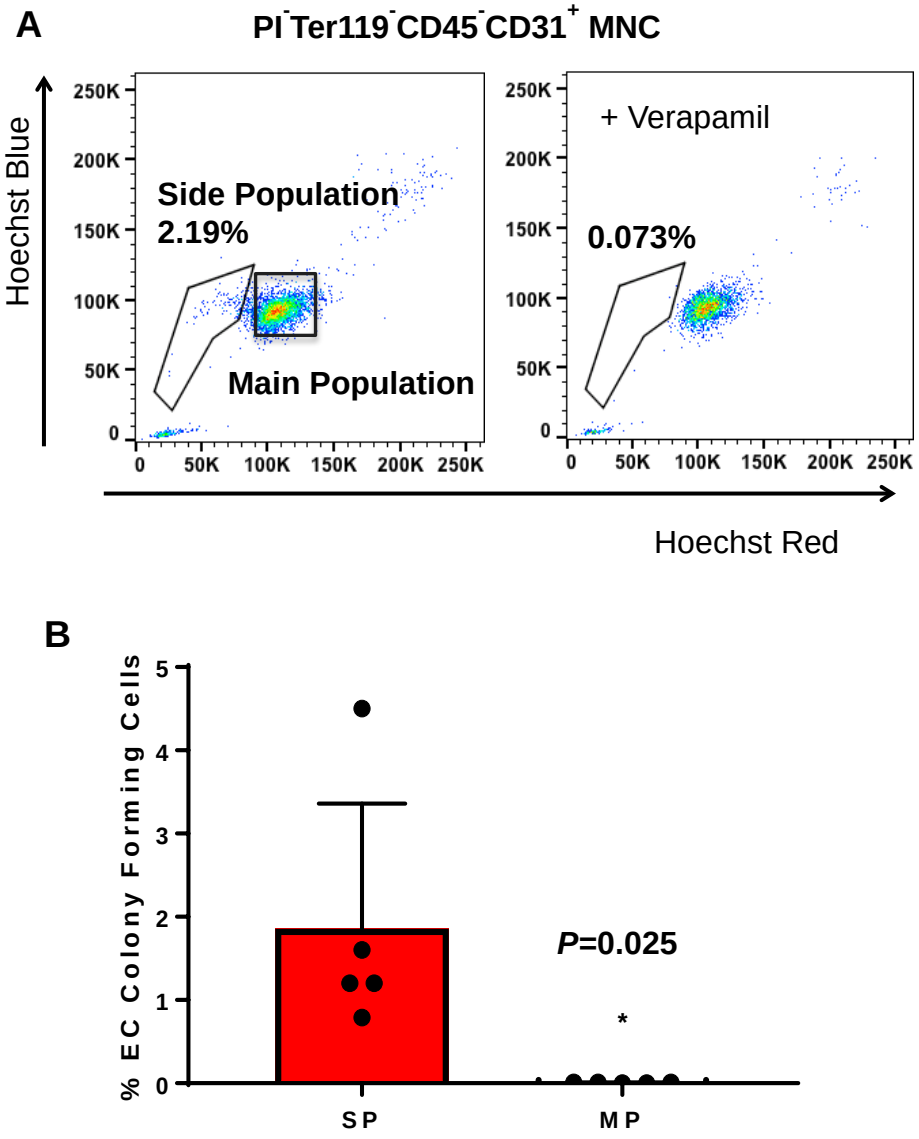


Figure II. 2. Endothelial colony forming cells are enriched in side populations in the adult mouse lung. (A). Representative flow cytometry chart showing side and main populations from 8 weeks old adult lung EC (left panel). Adding of verapamil, a calcium channel inhibitor, blocks SP phenotype (right panel). (B). Quantitation of the percentage of EC colony forming cells in lung EC ($CD45^{-}CD31^{+}$) side population (SP) and main population (MP). Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=5 mice).

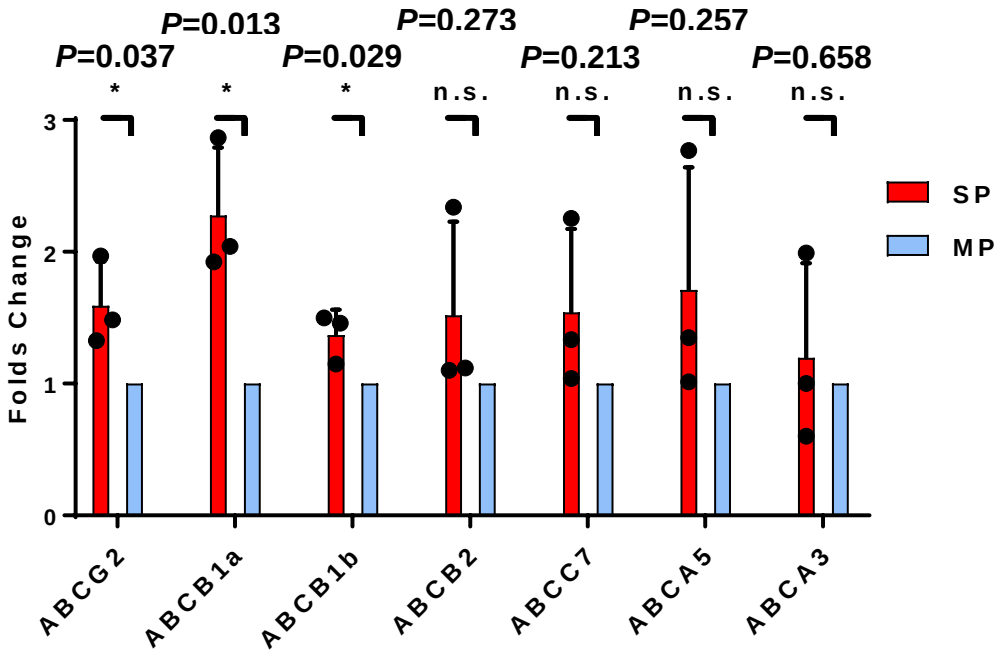
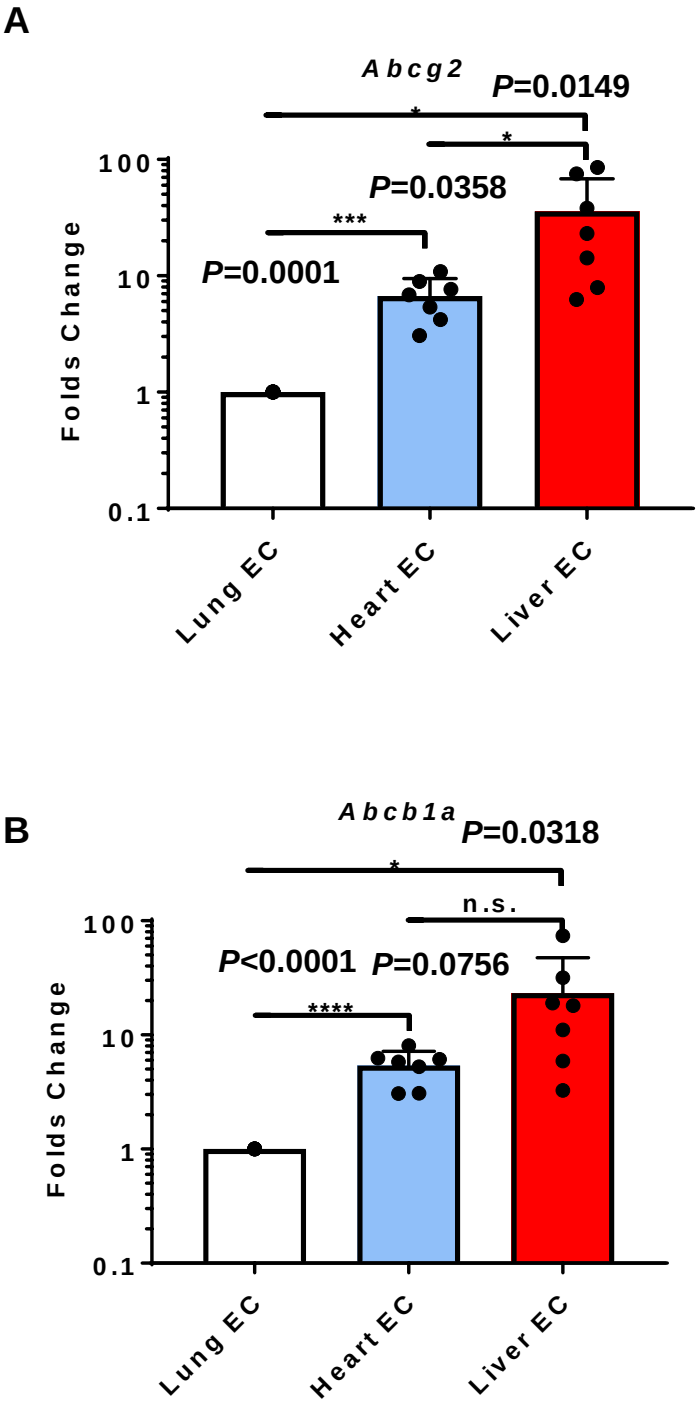
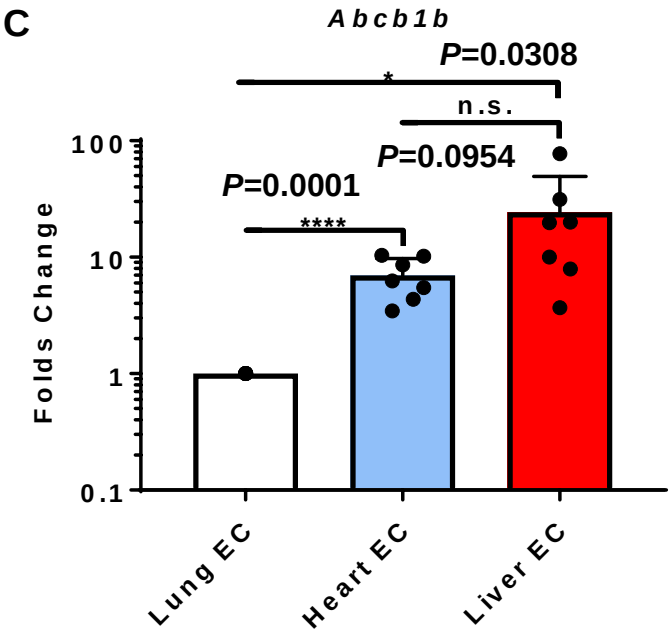


Figure II. 3. qPCR analysis of the expression of seven ATP binding cassette family transporters in lung CD45$^-$CD31$^+$ EC side population (SP, red bars) and main population (MP, blue bars) . Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=3 mice from 3 independent experiments).

Since *Abcg2* is known to be the molecular determinant of the SP in other tissue stem cell lineages (Zhou et al., 2002; Zhou et al., 2001), we examined the role of *Abcg2* in the emergence and maintenance of EC stem / progenitor cells. We first determined the level of expression of *Abcg2* in SP from vascular EC isolated from several different tissues and observed up to 80-fold differences in *Abcg2* expression comparing lung, liver, and heart EC (Figure II. 4 A-C). Thus, while the level of *Abcg2* expression cannot be correlated with the ECFC potential of EC from different tissues (Figure II. 4 D), the *in vi*tro block of Abcg2 function by the known inhibitor verapamil (Figure II. 5) or loss of *Abcg2* expression in *Abcg2* knockout (KO) mice (*n*=6) (Figure II. 6), resulted in a significant depletion of ECFC. This result is consistent with previously published data that the depletion of *Abcg2* affects the emergence, maintenance, and survival of numerous tissue stem/progenitor cells (Bhattacharya et al., 2007; Chen et al., 2010; Pfister et al., 2008; Zhou et al., 2002; Zhou et al., 2001; Zhou et al., 2003) and diminishes the ability of EC to replenish damaged blood vessels after cardiovascular tissue injuries (Doyle et al., 2011; Higashikuni et al., 2010; Higashikuni et al., 2012; Maher et al., 2014).


A
Abcg2
P=0.0149
*
*
P=0.0358
***
P=0.0001
100
10
1
0.1
Folds Change
Lung EC
Heart EC
Liver EC
B
Abcb1a
P=0.0318
*
n.s.
P<0.0001
P=0.0756
****
100
10
1
0.1
Folds Change
Lung EC
Heart EC
Liver EC


C
Abcb1b
P=0.0308
*
n.s.
P=0.0954
P=0.0001
****
100
10
1
0.1
Folds Change
Lung EC
Heart EC
Liver EC

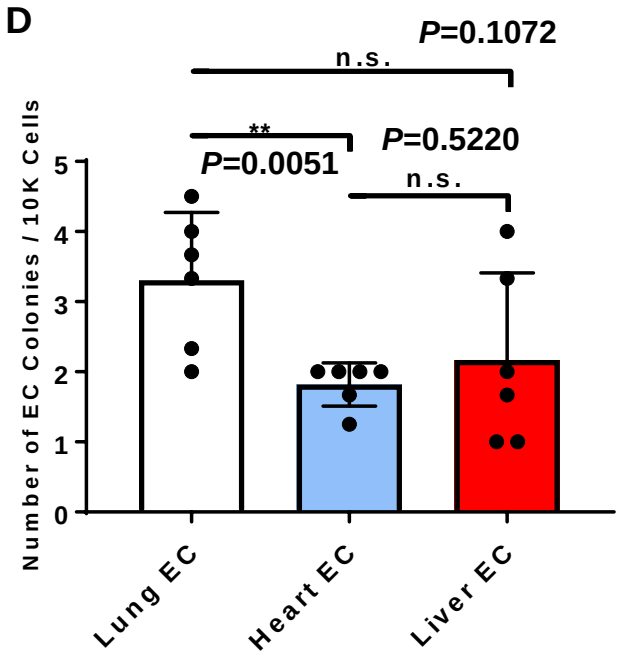
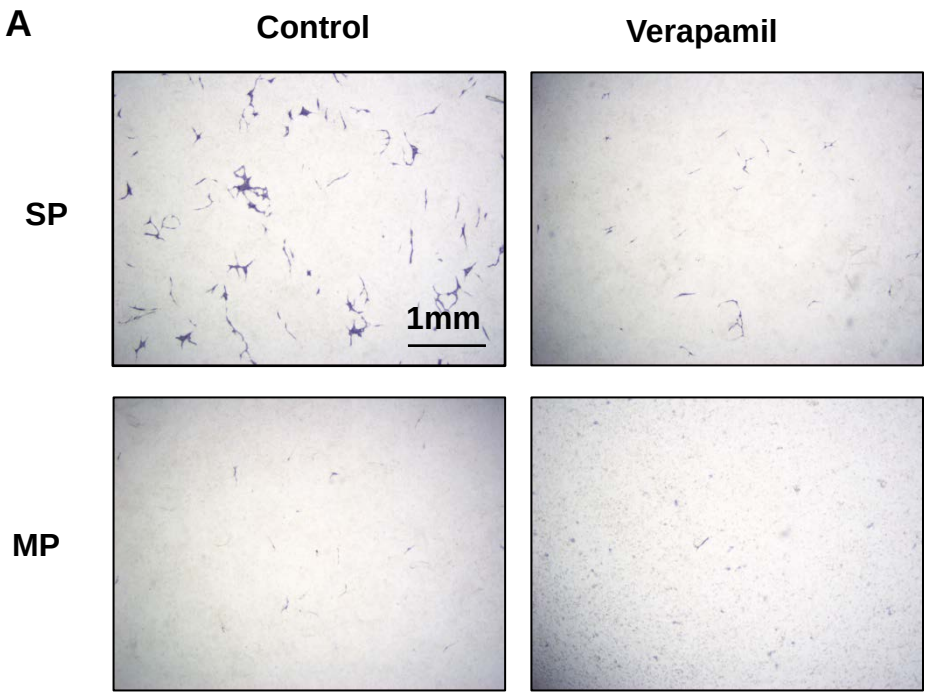

D
P=0.1072
n.s.
**
P=0.5220
P=0.0051
n.s.
5
4
3
2
1
0
Number of EC Colonies / 10K Cells
Lung EC
Heart EC
Liver EC

Figure II. 4. The expression of *Abcg2*, *Abcb1a*, *Abcb1b* is not correlated with the endothelial colony forming potential of each organ specific endothelial cells. (A-C). qPCR data showing the expression of *Abcg2* (A), *Abcb1a* (B) and *Abcb1b* (C) in 8 week old murine lung (white bar), heart (blue bar) and liver (red bar) EC. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=7 mice). (D). Frequency of colony forming cells in EC from 8 week old murine lung (white bar), heart (blue bar) and liver (red bar). Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=6 mice).


A
Control
Verapamil
SP
MP
1mm

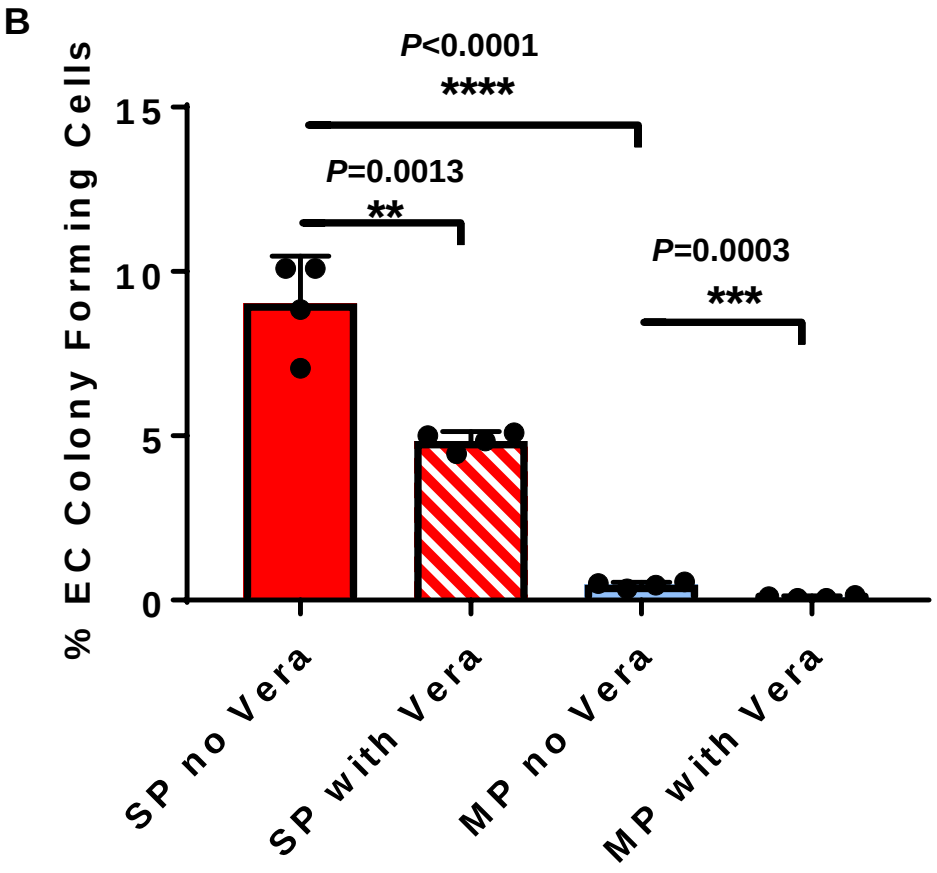
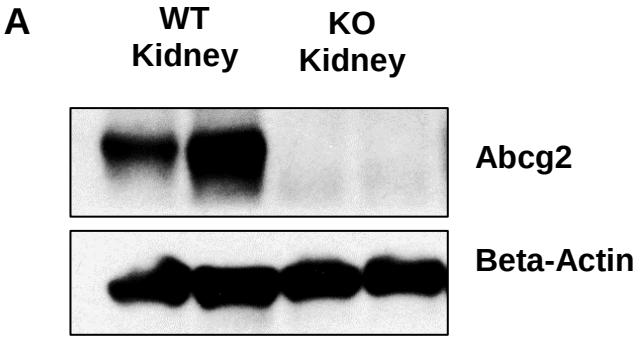
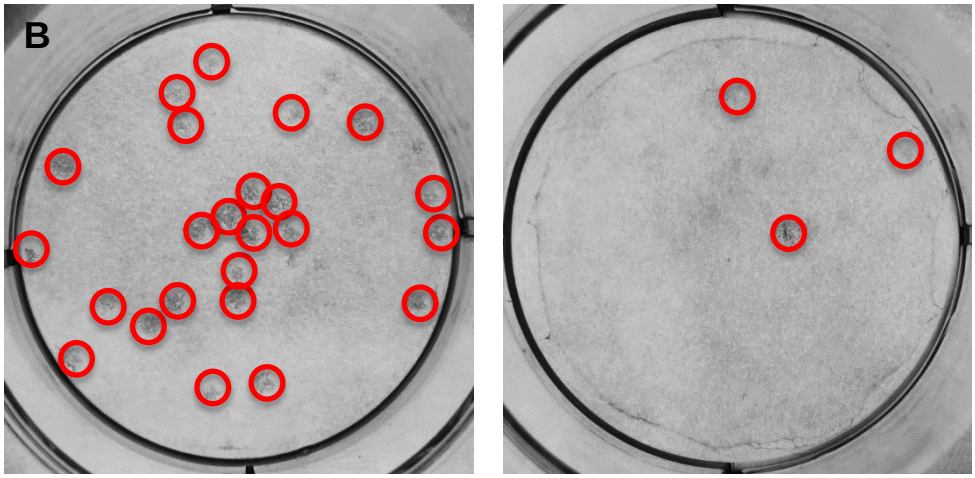

B
P<0.0001
****
P=0.0013
**
P=0.0003
***
% EC Colony Forming Cells
15
10
5
0
SP no Vera
SP with Vera
MP no Vera
MP with Vera

Figure II. 5. Verapamil blocks the growth of lung endothelial cells *in vitro*. (A). Representative picture of side population (SP) and main population (MP) EC from 8 weeks old mice cultured on OP9 with or without verapamil. (B). Quantitation of colony forming potential of 8 weeks old lung EC side population (SP no Vera, red bar), side population with verapamil (SP with Vera, red stripes bar), main population (MP no Vera, blue bar) and main population with verapamil (MP with Vera, blue stripes bar). Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=4 mice).


A
WT Kidney
KO Kidney
Abcg2
Beta-Actin


B
WT
KO

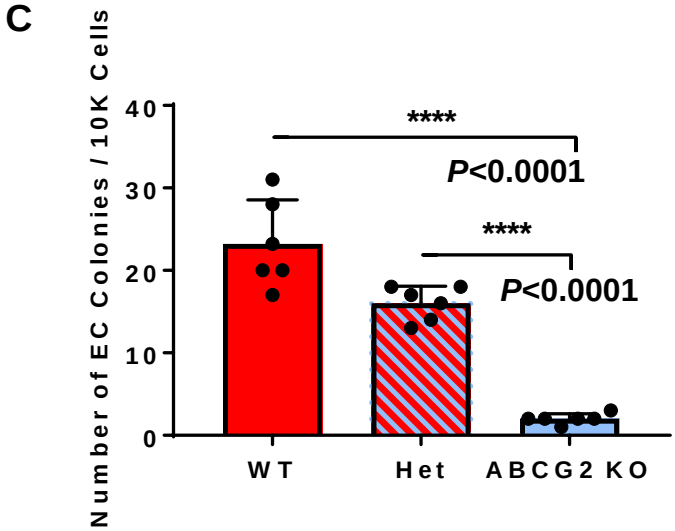
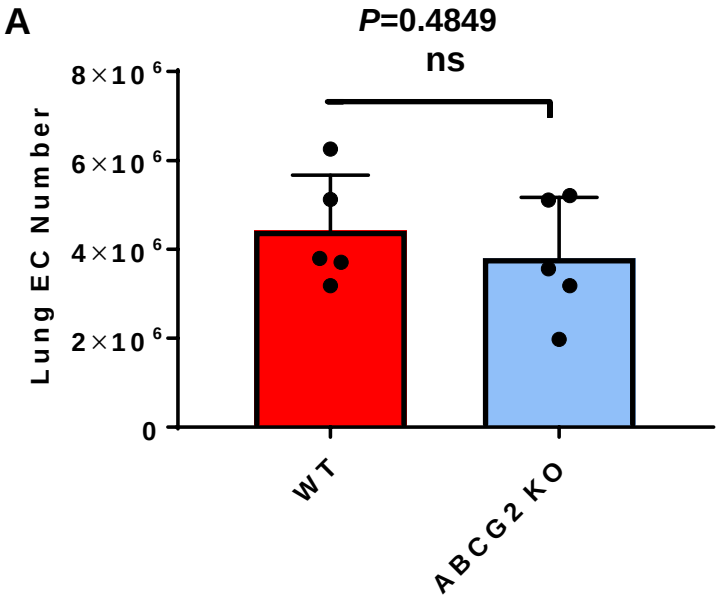
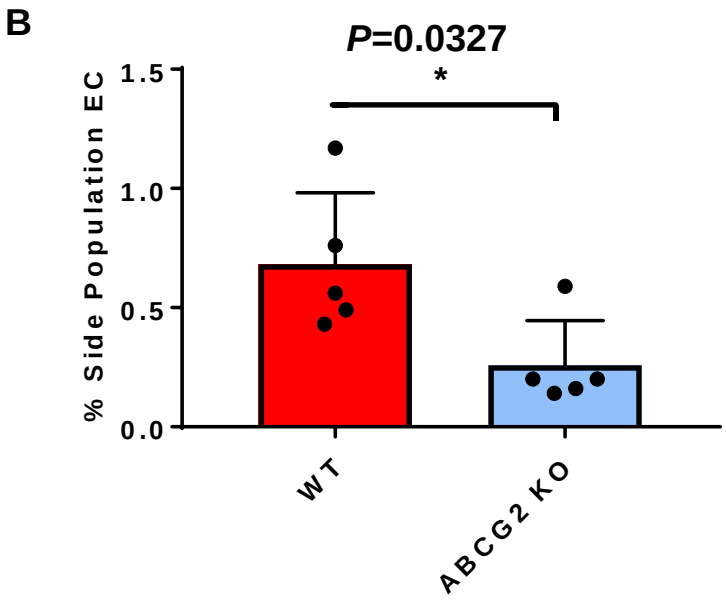

C
Number of EC Colonies / 10K Cells
40
30
20
10
0
****
P<0.0001
****
P<0.0001
WT
Het
ABCG2 KO

Figure II. 6. Abcg2 is crucial for the maintenance of EC colony forming cells. (A). Western blot of Abcg2 in the kidneys of wild type (WT) and *Abcg2* knockout (KO) mice. Beta actin was used as internal control. (B). Representative pictures of $CD31^+$ EC colonies (indicated by red circles) derived from 10, 000 lung $CD45^-CD31^+$ EC (OP9 co-culture, plated in 1 well of 6 well plate) from P1 wild type FVB (WT, left panel) and *Abcg2* knockout (KO, right panel) mice. (C). Quantitation of numbers of EC colony forming EC in 10, 000 (10K) lung $CD45^-CD31^+$ EC from P1 wild type FVB (WT, red bar), *Abcg2* knockout (KO, blue bar) and heterozygous (Het, red stripes bar) mice. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=6 mice from 2 independent experiments).

Although the frequency of SP EC was dramatically diminished in KO mice (Figure II. 7), colony-forming EC were still enriched in the minimal residual SP fraction of KO mice (Figure II. 8). We reasoned that retention of ECFC in the diminished SP population was caused by a compensatory up-regulation of other ABC family transporters, including *Abcb1a* and *Abcb1b* in the KO EC SP fraction (Figure II. 9). To test that hypothesis, tetrandrine, a known specific inhibitor for *Abcb1a* and *Abcb1b* (Fanelli et al., 2016; Shen et al., 2010) was injected into *Abcg2* KO pups for 4 days (from postnatal day [P] 0 through P3) and the treated pups (n=5) were analyzed for ECFC formation from vascular EC (Figure II. 10A). A significant further decline in the number of EC SP, ECFC frequency, and total ECFC number in the lung (Figure II. 10B-E) was detected. These data indicate that *Abcg2* is the main determinant of ECFC emergence, maintenance, and SP phenotype, while *Abcb1a* and *Abcb1b* play supporting roles in vascular ECFC emergence / maintenance.


A
P=0.4849
ns
Lung EC Number
8×10 6
6×10 6
4×10 6
2×10 6
0
WT
ABCG2 KO


B
P=0.0327
*
% Side Population EC
1.5
1.0
0.5
0.0
WT
ABCG2 KO

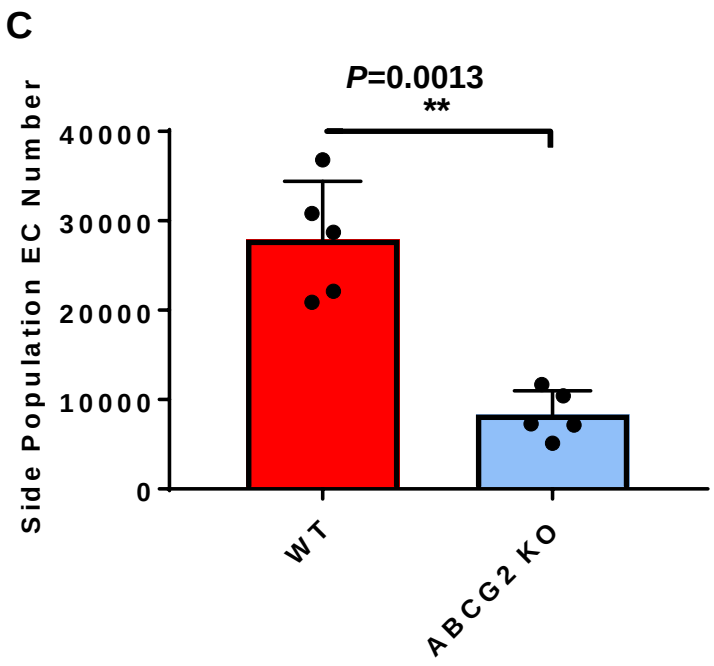


Figure II. 7. Abcg2 is crucial for the maintenance of side population phenotype. Numbers of lung EC (A), percentage of lung EC side population (B) and numbers of lung EC side population (C) from P8 wild type FVB (WT, red bar) and *Abcg2* knockout (ABCG2KO, blue bar) mice. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=5 mice from 2 independent experiments).

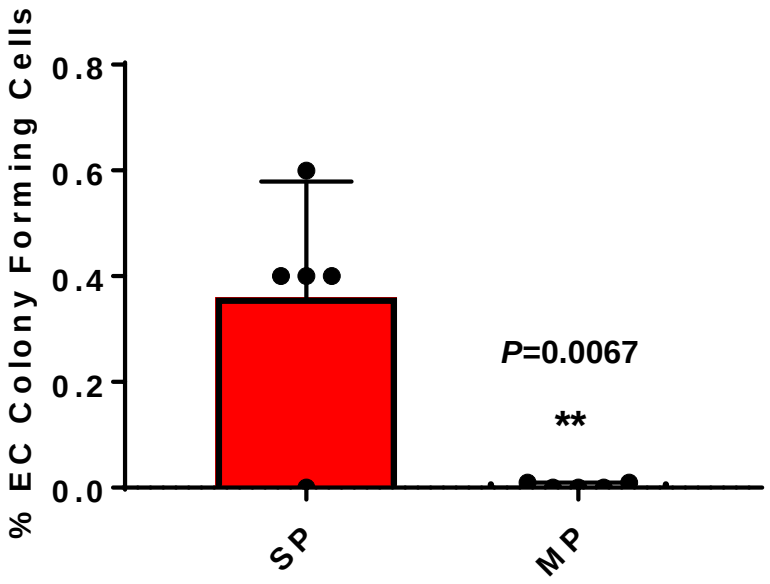


Figure II. 8. Frequency of colony forming EC in side population (SP, red bar) and main population (MP, blue bar) from P8 *Abcg2* knockout mice lung EC. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=5 mice).

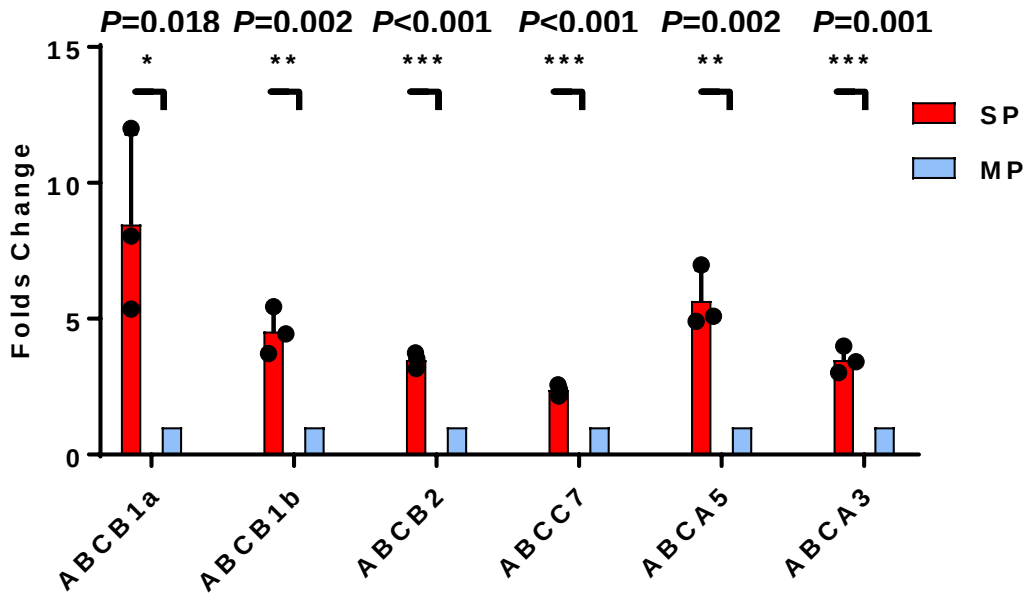
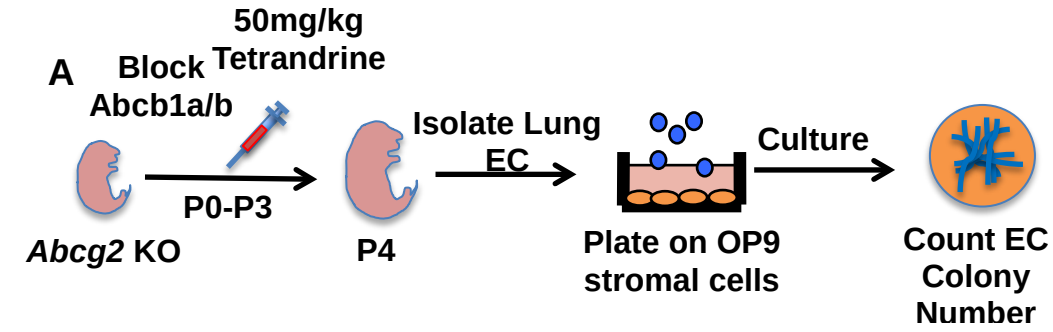
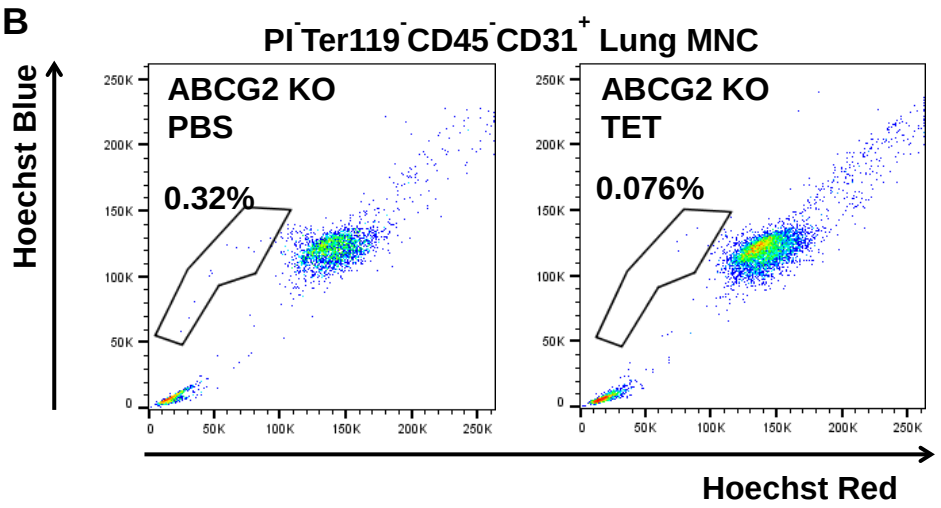
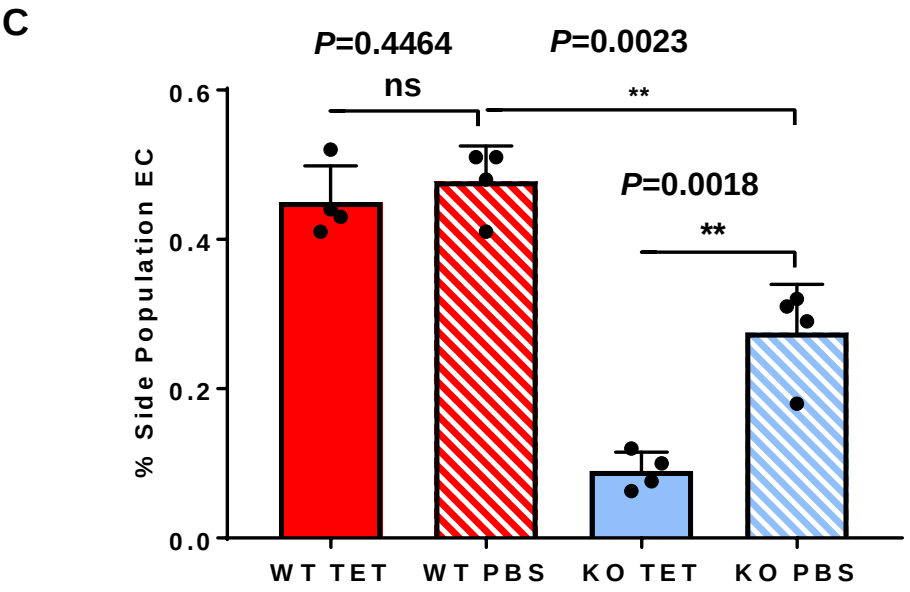


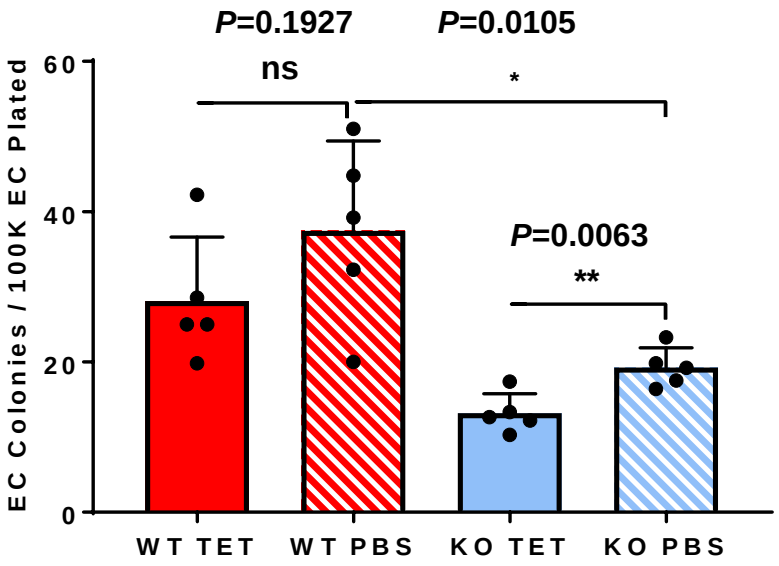
Figure II. 9. qPCR analysis of the expression of six ATP binding cassette family transporters in lung CD45$^-$CD31$^+$ EC side population (SP, red bars) and main population (MP, blue bars) from *Abcg2* knockout mice. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=3 mice from 3 independent experiments).


A
Block Abcb1a/b
50mg/kg Tetrandrine
P0-P3
Abcg2 KO
P4
Isolate Lung EC
Culture
Plate on OP9 stromal cells
Count EC Colony Number


B
PI- Ter119- CD45- CD31+ Lung MNC
Hoechst Blue
ABCG2 KO PBS
0.32%
ABCG2 KO TET
0.076%
Hoechst Red


C
P=0.4464
ns
P=0.0023
**
P=0.0018
**
% Side Population EC
0.6
0.4
0.2
0.0
WT TET
WT PBS
KO TET
KO PBS

D


P=0.1927
ns
P=0.0105
*
P=0.0063
**
EC Colonies / 100K EC Plated
60
40
20
0
WT TET
WT PBS
KO TET
KO PBS

E

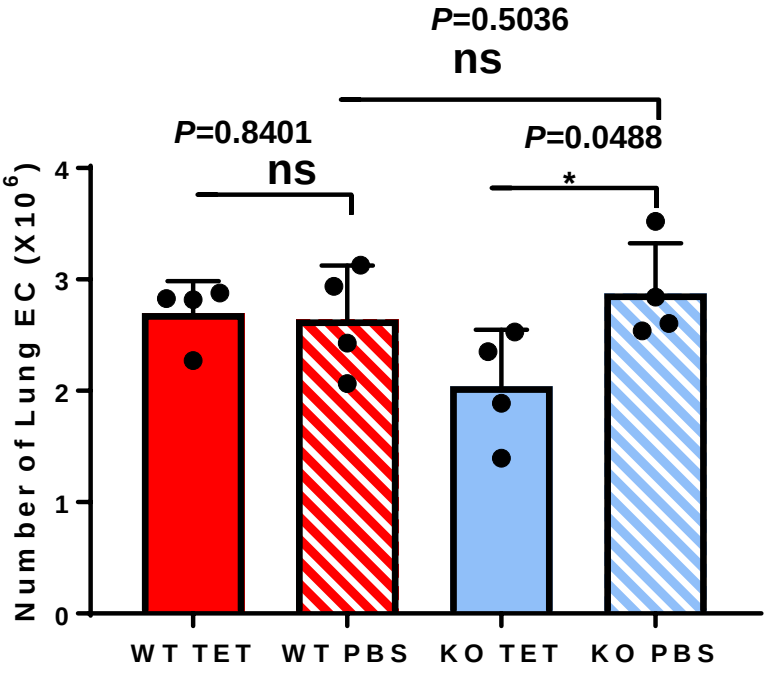

P=0.5036
ns
P=0.8401
ns
P=0.0488
*
Number of Lung EC (X10^6)
4
3
2
1
0
WT TET
WT PBS
KO TET
KO PBS

Figure II. 10. *Abcb1a* and *Abcb1b* play supporting roles in vascular ECFC emergence / maintenance. (A). Experiment schematic for tetrandrine's effect on colony forming EC in *Abcg2* knockout mice. (B). Representative flow cytometry data shows P4 *Abcg2* knockout mice lung EC side population with 3 days treatment of PBS (ABCG2KO PBS, left panel) or tetrandrine (ABCG2KO TET, right panel). (C) – (E). Quantitation for lung EC side population percentage (C), frequency of colony forming EC (D) and lung EC number (E) in P4 wild type FVB with 3 days treatment of terandrine (WT TET, red bar) or PBS (WT PBS, red stripes bar), or *Abcg2* knockout mice with the treatment of terandrine (KO TET, blue bar) or PBS (KO PBS, blue stripes bar). Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (k and m, *n*=4 mice; l, *n*=5 mice).

Murine blood vessels contain *Abcg2*-expressing VESC with EC colony forming potential and *in vivo* vessel forming potential

Because *Abcg2* expression was important for the maintenance of vascular ECFC, we reasoned that the expression of *Abcg2* may be useful to identify putative endothelial stem cells upstream of the ECFC in the vascular endothelium. By breeding mice transgenic for a tamoxifen inducible *Abcg2* promoter driven Cre recombinase (*Abcg2CreERT2* (Fatima et al., 2012)) with *ROSATdTomato* mice, we generated ABCG2TT mice to identify the distribution and proliferation/differentiation potential of *Abcg2*-expressing EC in the murine system. (Figure II. 11). First, to survey the distribution of *Abcg2*-expressing EC during development, ABCG2TT pups were injected with 50mg/kg body weight tamoxifen on P0. After 24 hours, a small fraction of EC in multiple tissues of P1 pups were labeled with TdTomato (8.1±4.1% in heart EC, 0.5±0.09% in lung EC, 3.4±0.9% in bone marrow EC, *n*=5, Figure II. 12), while tamoxifen injected wild type ROSATdTomato mice (TT) displayed no TdTomato$^+$ cells (Figure II. 12).

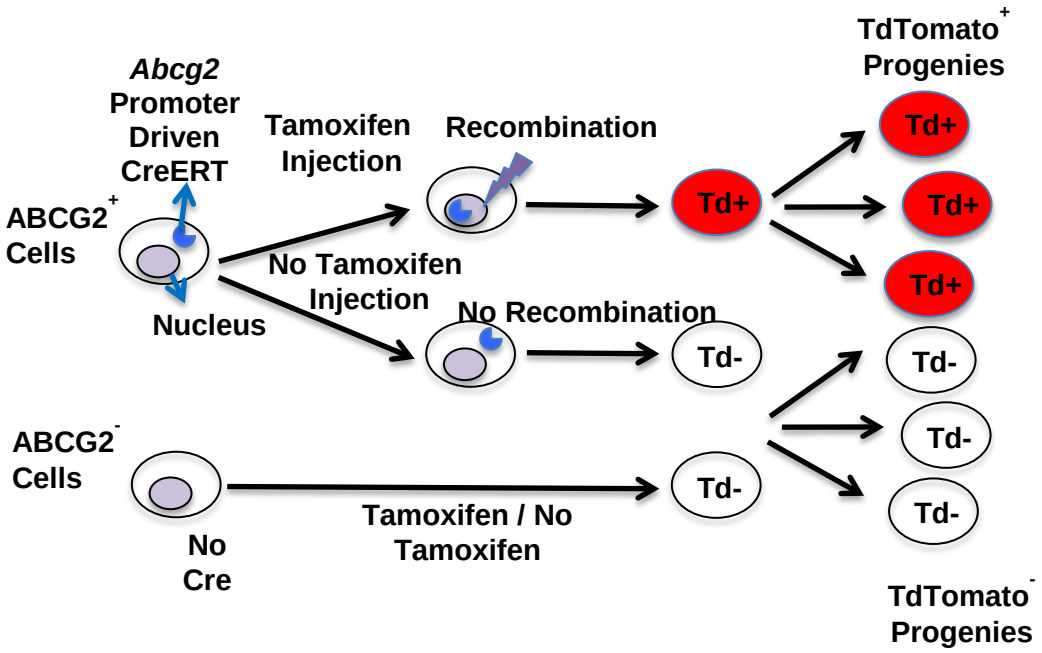


Figure II. 11. *Schematics* of lineage tracing experiments using ABCG2TT mice.

From P1 PI$^-$CD45$^-$Ter119$^-$:

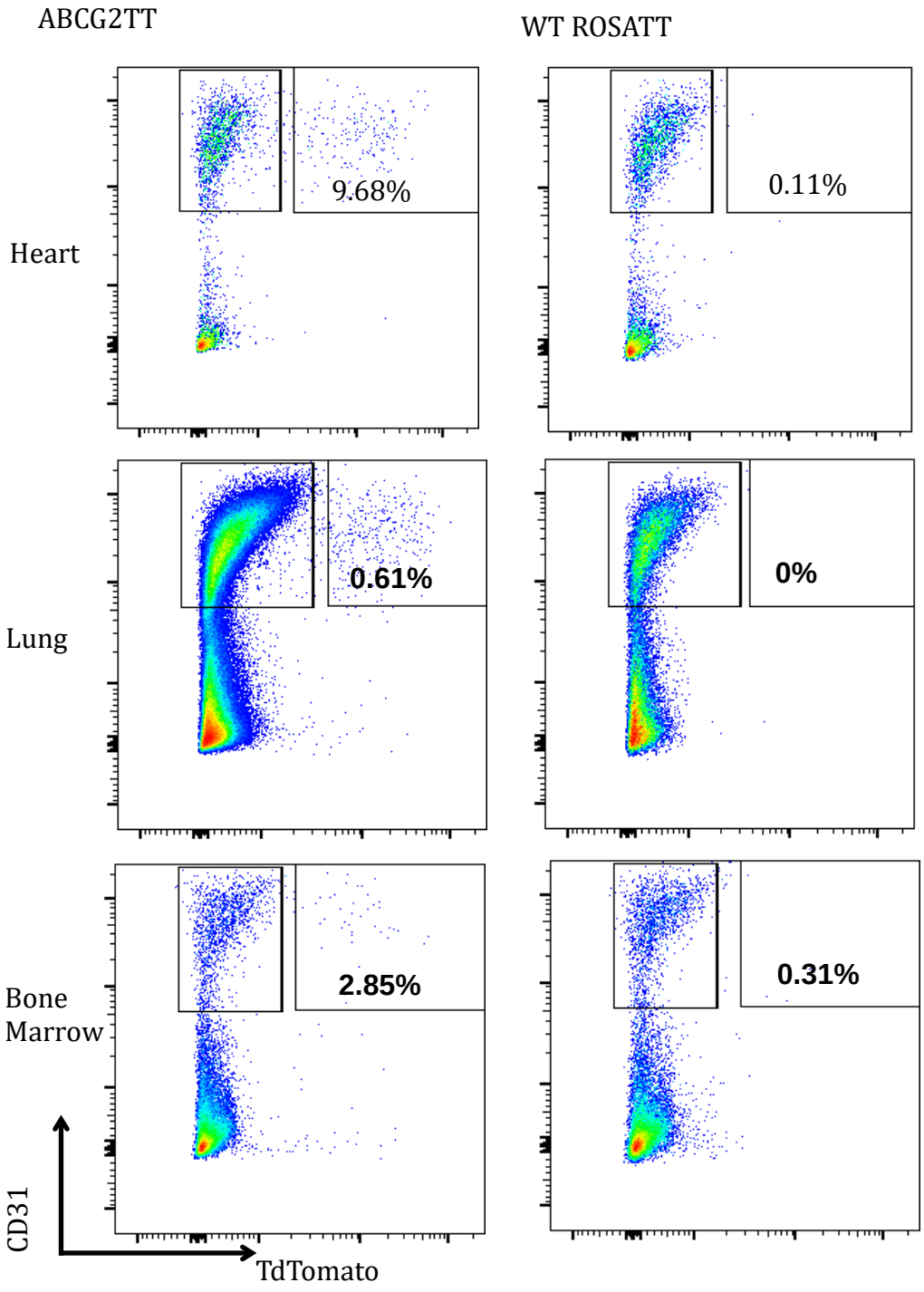
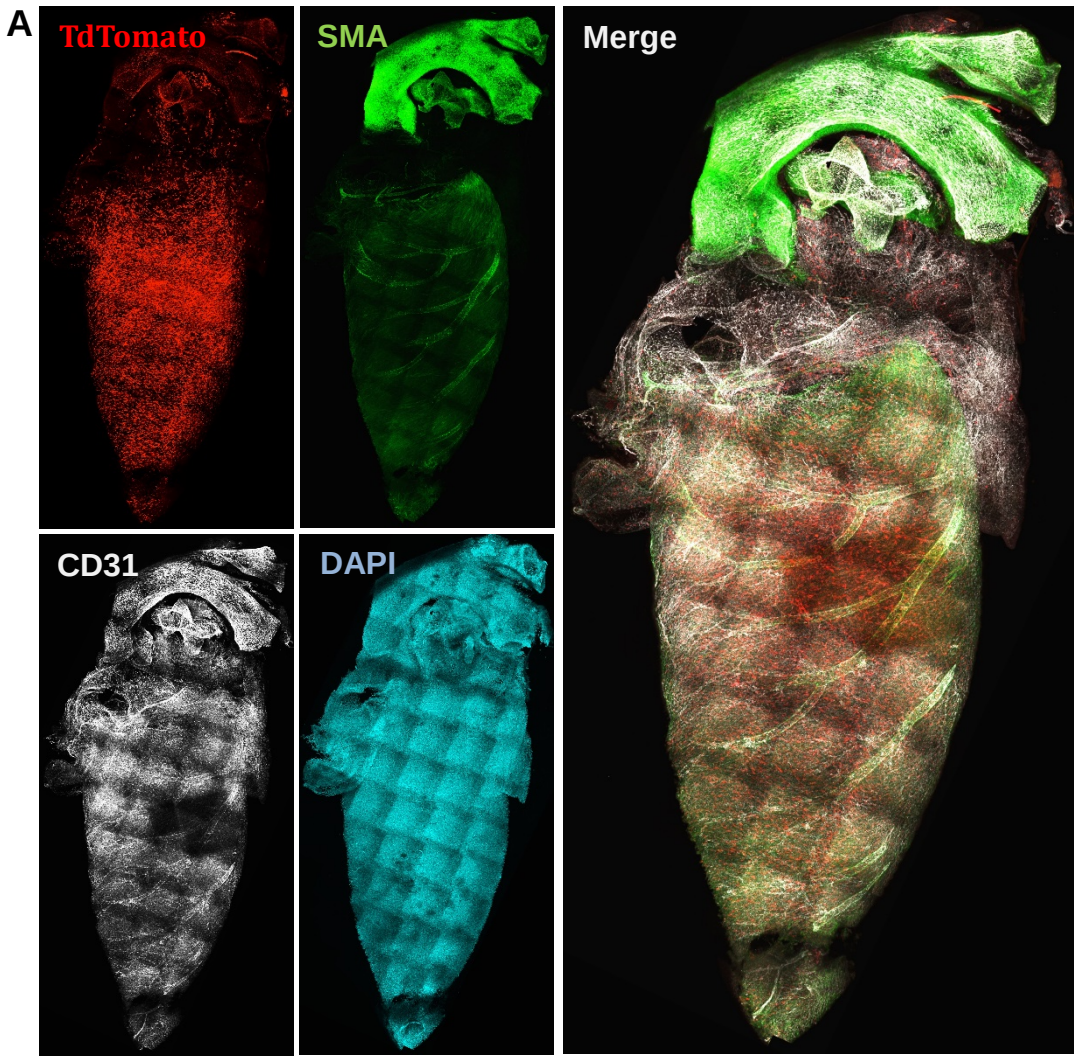


Figure II. 12. Representative flow cytometry data shows the frequency of TdTomato^{+} EC in P1 ABCG2TT mice heart, lung and bone marrow EC after tamoxifen injection at P0.

Microscopic images of tissues from pups sacrificed on P1 revealed that TdTomato$^+$ EC were distributed in multiple tissue blood vessels include heart (Figure II. 13), lung (Figure II. 14), bone (Figure II. 15), and skeletal muscle (Figure II. 15). In addition, *Abcg2*-expressing TdTomato$^+$ EC could be found in arteries (covered by thick smooth muscle layer [strong smooth muscle actin α](Sorensen et al., 2009; Townsley, 2012)), veins (diameter >20μm, covered by thin smooth muscle layer [weak or no smooth muscle actin α](Sorensen et al., 2009; Townsley, 2012)) and capillaries (diameter <10μm(Townsley, 2012)) (Figure II. 13, 14).


A
TdTomato
SMA
Merge
CD31
DAPI

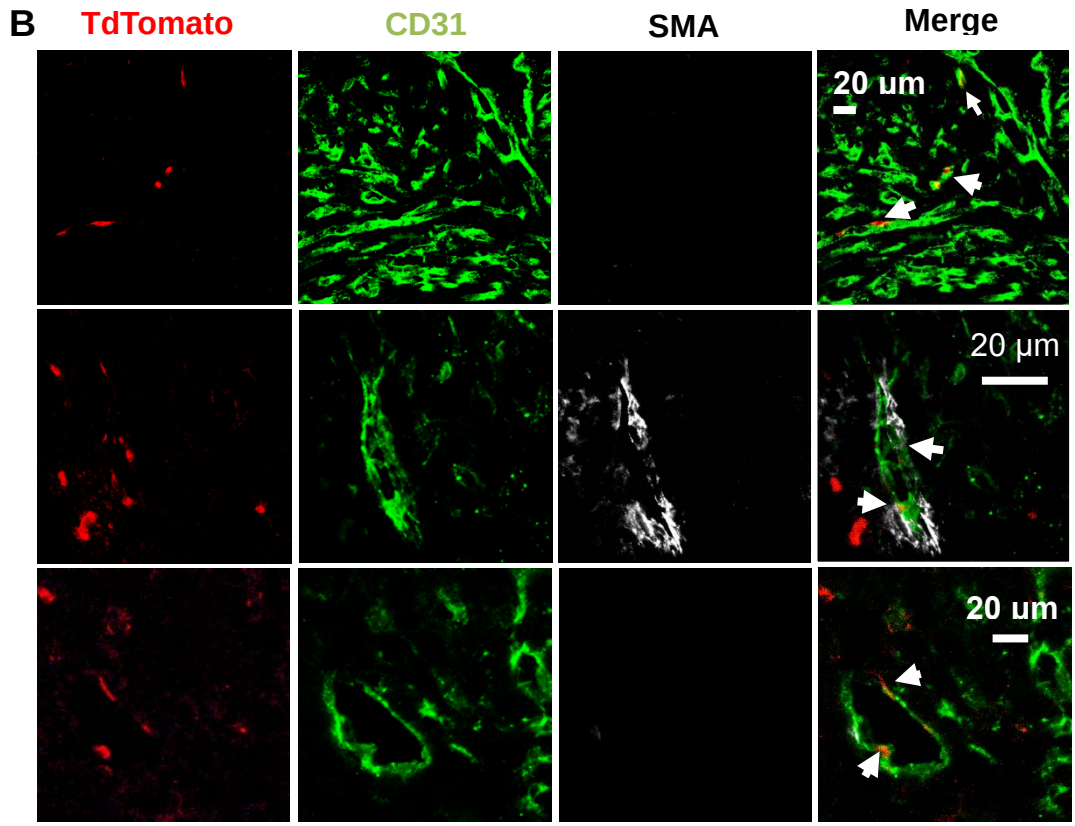


Figure II. 13. *Abcg2*-expressing EC are distributed in the heart of developing mice. (A). Deep imaging of a P1 ABCG2TT heart shows the distribution of TdTomato$^+$ (red), smooth muscle actin α$^+$ (SMA), CD31$^+$ (gray) cells and DAPI (blue). (B). Representative pictures show the distribution of TdTomato$^+$ EC in the capillaries (top panels, arrows), artery (middle panels, arrows) and vein (bottom panels, arrows) of a P1 ABCG2TT mouse heart after tamoxifen injection at P0 (Red, TdTomato, Green, CD31, Gray, smooth muscle actin α$^+$ [SMA]).

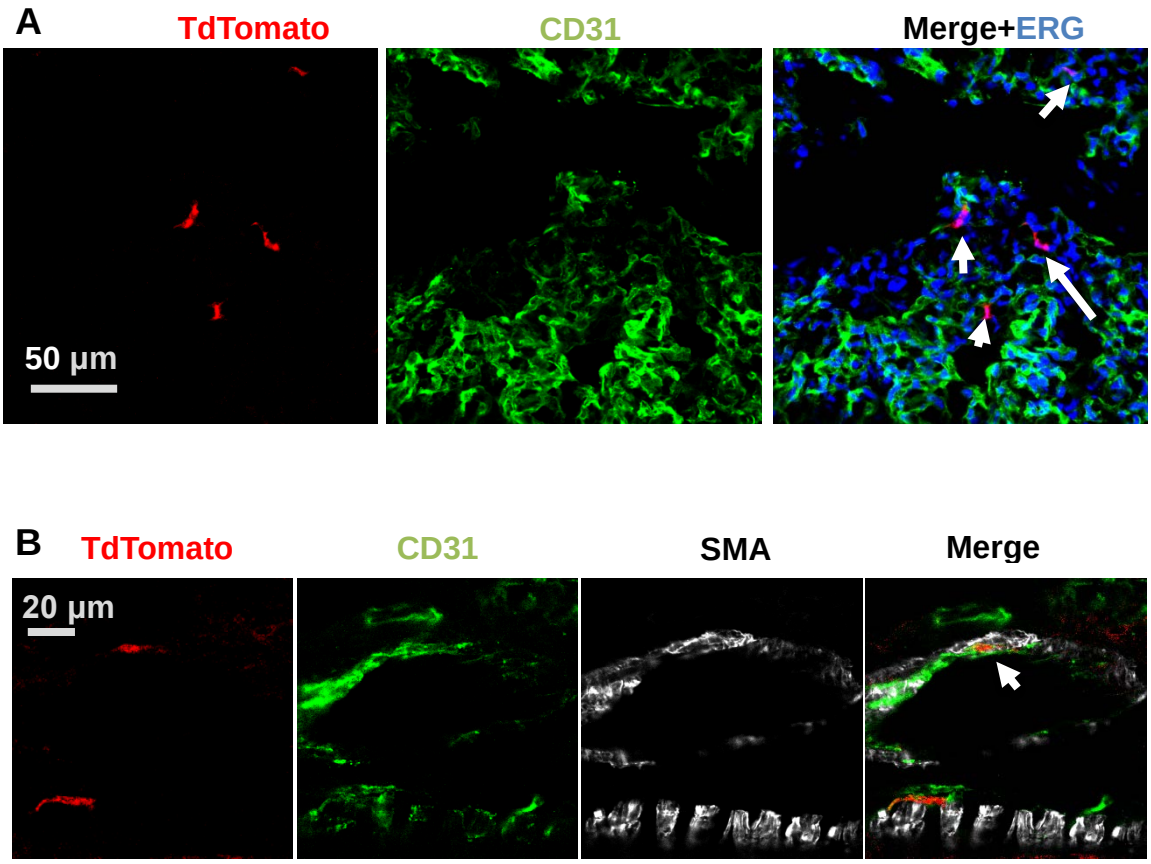


Figure II. 14. *Abcg2*-expressing EC are distributed in the lung of developing mice. Representative pictures of TdTomato⁺ EC in P1 ABCG2TT mouse lung capillaries (A, arrows) and artery (B, arrows) after tamoxifen injection at P0.

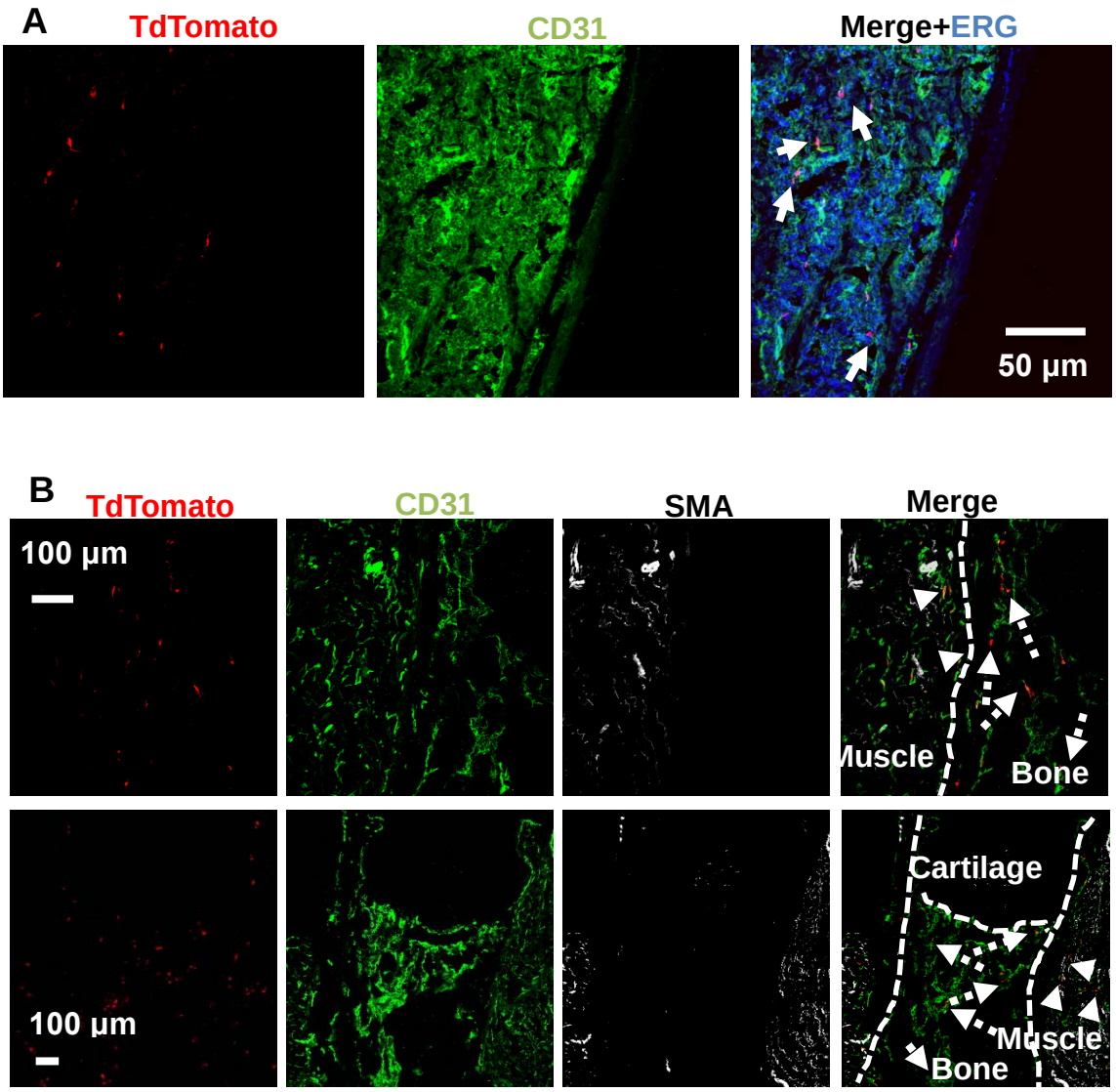
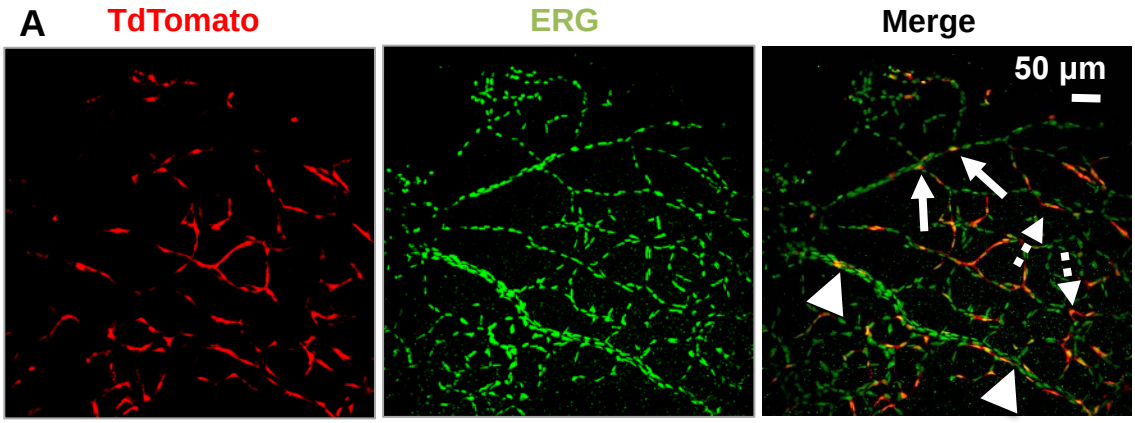
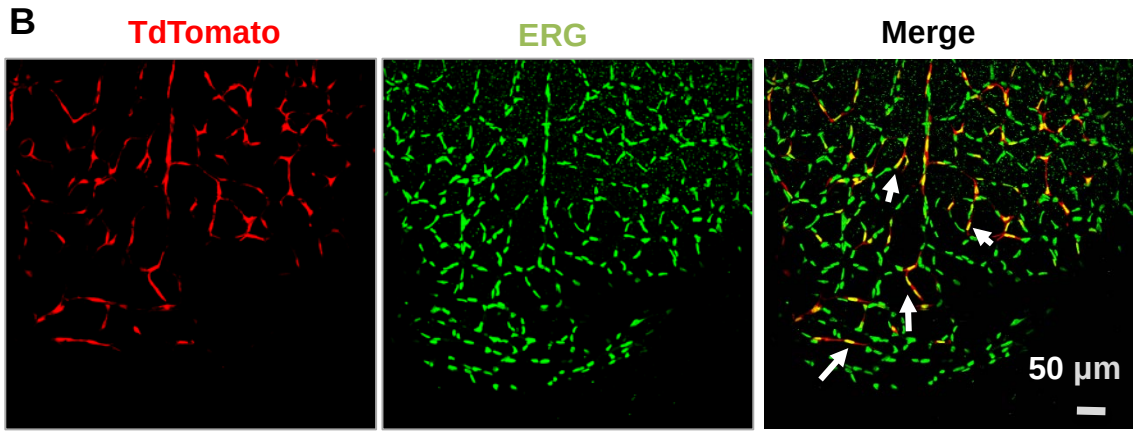
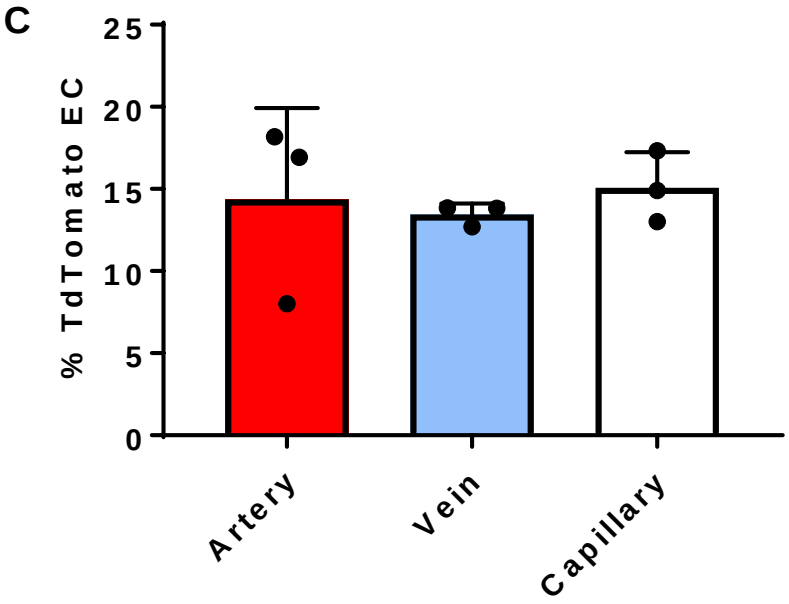


Figure II. 15. *Abcg2*-expressing EC is distributed in the bone marrow and skeletal muscle of developing mice. (A). Representative pictures of TdTomato$^+$ EC in P1 ABCG2TT bone marrow blood vessels (arrows) after tamoxifen injection at P0. (B) Representative pictures of TdTomato$^+$ EC in P1 ABCG2TT bone marrow (dashed arrows) and skeletal muscle (arrows) blood vessels after tamoxifen injection at P0. From a-d, Red: TdTomato, Green, CD31, Blue, ERG.

To investigate the distribution of *Abcg2*-expressing EC in arteries, veins and capillaries in neonatal retina, we injected tamoxifen at P3 and analyzed the pups at P4, a time when arteries and veins are first morphologically identifiable (Stalmans et al., 2002; Uemura et al., 2006). Similar to other developing tissues, P4 retinal vessels displayed TdTomato$^+$ EC in newly differentiated arteries (14.4±5.6%), veins (13.5±0.7%), and capillaries (15.1±2.2%) (*n*=3, Figure II. 16 A-C). In addition, TdTomato$^+$ EC were identified as tip, stalk, or phalanx cells in the growing retinal vascular beds (Figure II. 16 D). The majority of TdTomato$^+$ EC after 24 hours of tamoxifen injection were single cells and not grouped as cell clusters (Figure II. 13-16), suggesting at this time, most TdTomato$^+$ EC were *Abcg2*-expressing EC precursors, and not their progeny.


A
TdTomato
ERG
Merge
50 µm


B
TdTomato
ERG
Merge
50 µm


C
% TdTomato EC
25
20
15
10
5
0
Artery
Vein
Capillary

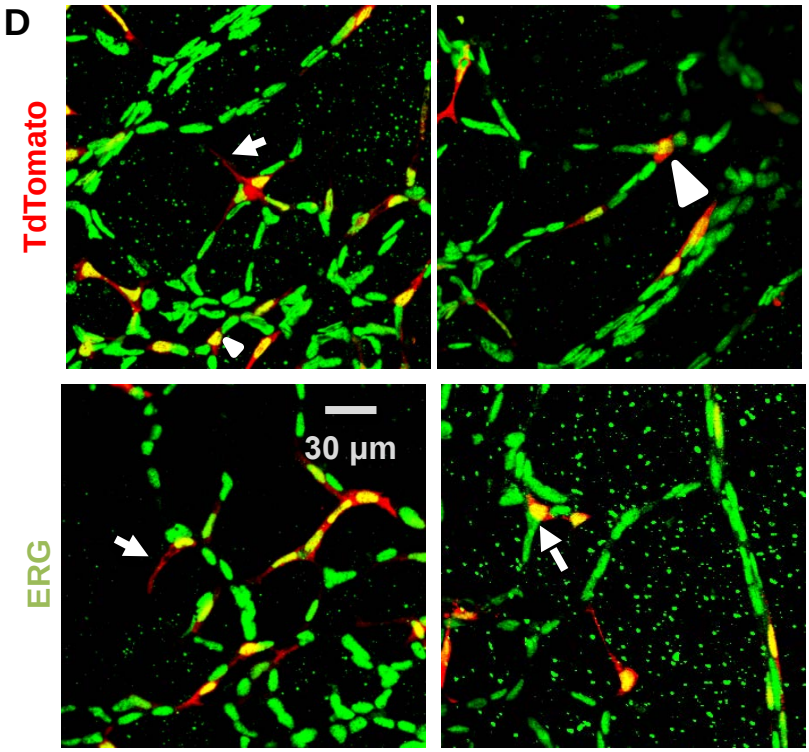

D
TdTomato
ERG
30 µm

Figure II. 16. *Abcg2*-expressing EC is distributed in the retina of developing mice. (A). **,** Representative pictures show TdTomato$^+$ EC in the artery (arrows), vein (arrowheads) and capillaries (dashed arrows) in P4 ABCG2TT mice retina after tamoxifen injection at P3. (B) Representative pictures of TdTomato$^+$ EC in P4 ABCG2TT retina capillaries (arrows) after tamoxifen injection at P0 (C). Quantitation of the percentage of TdTomato$^+$ EC in the artery (red bar), vein (blue bar) and capillaries (white bar) in P4 ABCG2TT mice retina after tamoxifen injection at P3. Data represent mean ± s. d. (n=3 mice. For each mice, ERG$^+$ nucleus of >200 arterial EC, >200 venous EC and >500 capillary EC from confocal images were analyzed). (D). Representative pictures of TdTomato$^+$ EC in P4 ABCG2TT retina tip (arrows), stalk (dashed arrows) and phalanx (arrowheads) cells after tamoxifen injection at P3. Red, TdTomato, Green, ERG

Similar to EC SP cells, TdTomato$^+$ EC were more highly enriched in *Abcg2*, *Abcb1a,* and *Abcb1b* transcripts than TdTomato$^-$ EC (Figure II. 17). To test if these cells represent endothelial stem cells, TdTomato$^+$ and TdTomato$^-$ EC from P1 heart and lung vessels were isolated by flow cytometry and were co-cultured over a monolayer of OP9 stromal cells (Figure II. 18A). After 10 days of co-culture, TdTomato$^+$ EC displayed significantly greater ECFC potential than TdTomato$^-$ EC (Figure II. 18B-D). Although at P1 only a small fraction of EC are TdTomato$^+$ (8.1±4.1% in heart EC and 0.5±0.09% in lung EC, *n*=5 mice, Figure II. 11), 49.2±7.4% of ECFC derived from heart and 16.7±4.7% of the lung ECFC were TdTomato$^+$ (*n*=4 mice, Figure II. 18E).

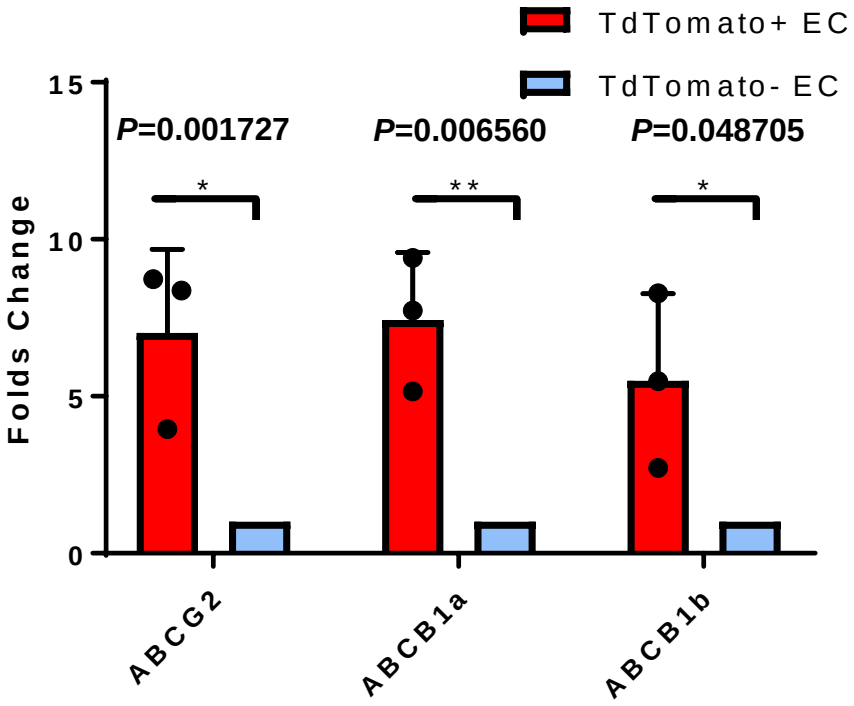
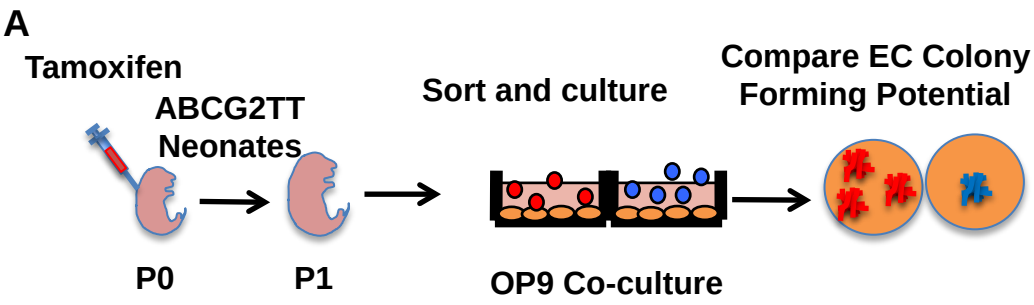
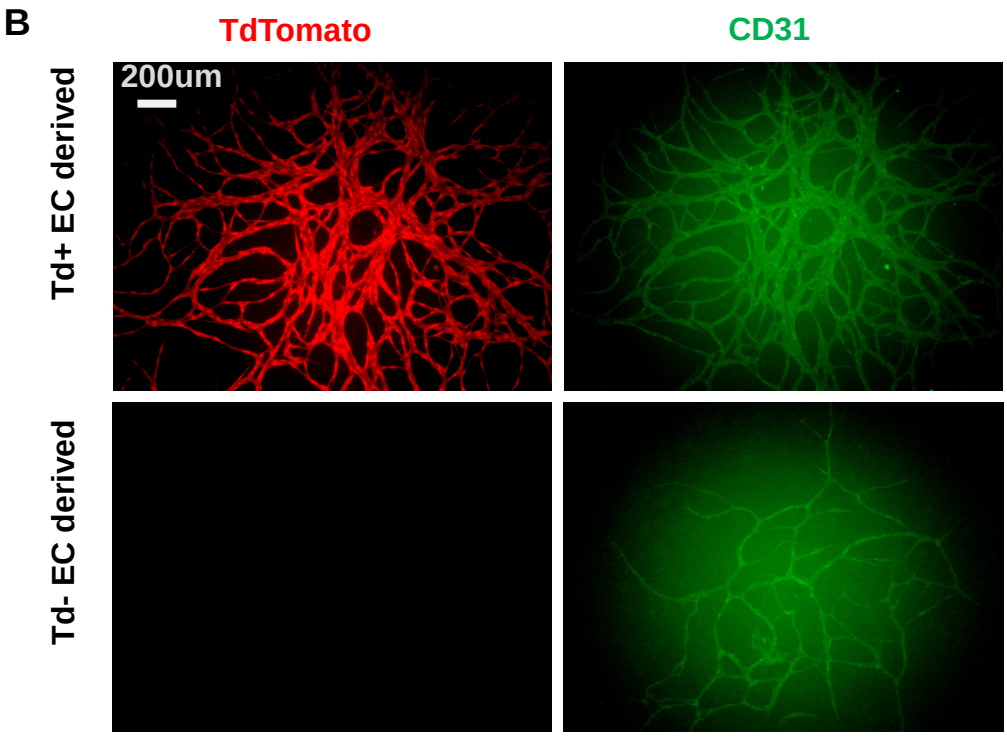
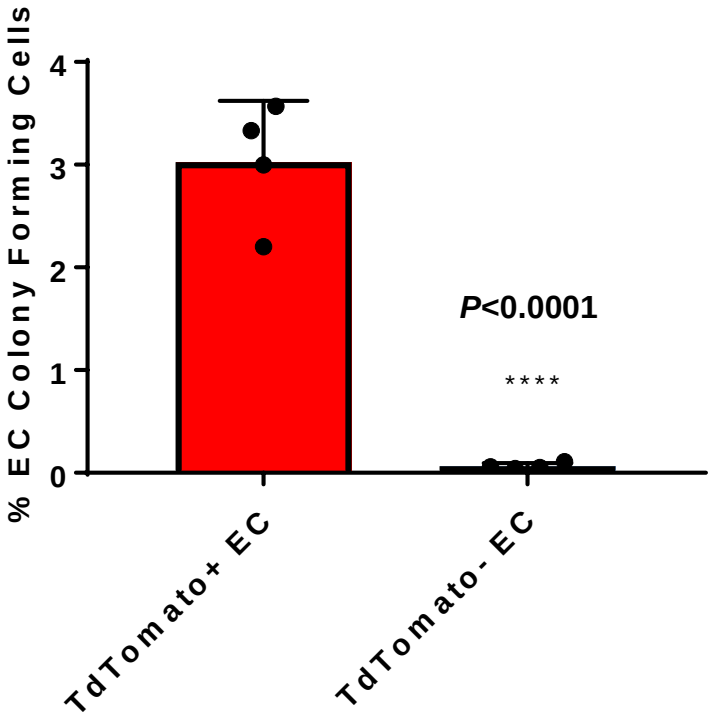


Figure II. 17. qPCR of the expression of *Abcg2*, *Abcb1a*, *Abcb1b* in P1 ABCG2TT mice heart TdTomato^{+} (red bars) and TdTomato^{-} (blue bars) EC (tamoxifen injection at P0). Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=3 mice).

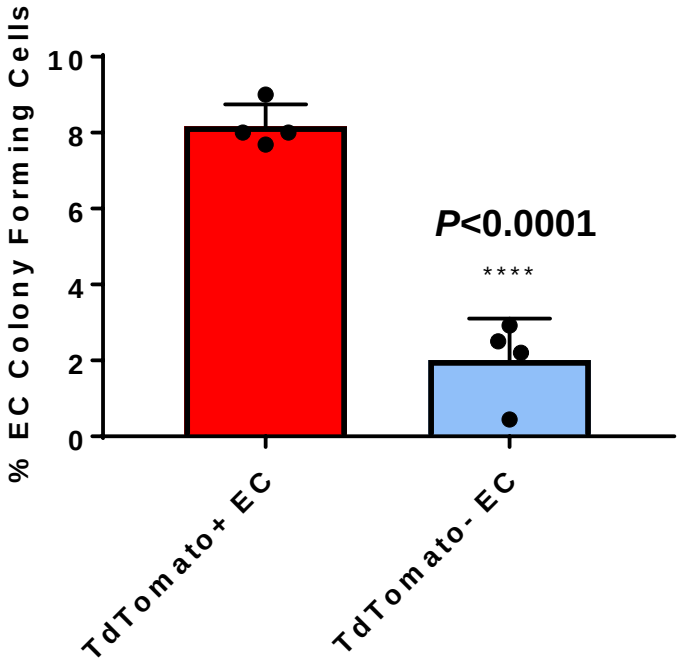

A
Tamoxifen
ABCG2TT
Neonates
P0
P1
Sort and culture
OP9 Co-culture
Compare EC Colony
Forming Potential


B
TdTomato
CD31
200um
Td+ EC derived
Td- EC derived

C


% EC Colony Forming Cells
4
3
2
1
0
P<0.0001
****
TdTomato+ EC
TdTomato- EC

D


% EC Colony Forming Cells
10
8
6
4
2
0
P<0.0001
****
TdTomato+ EC
TdTomato- EC

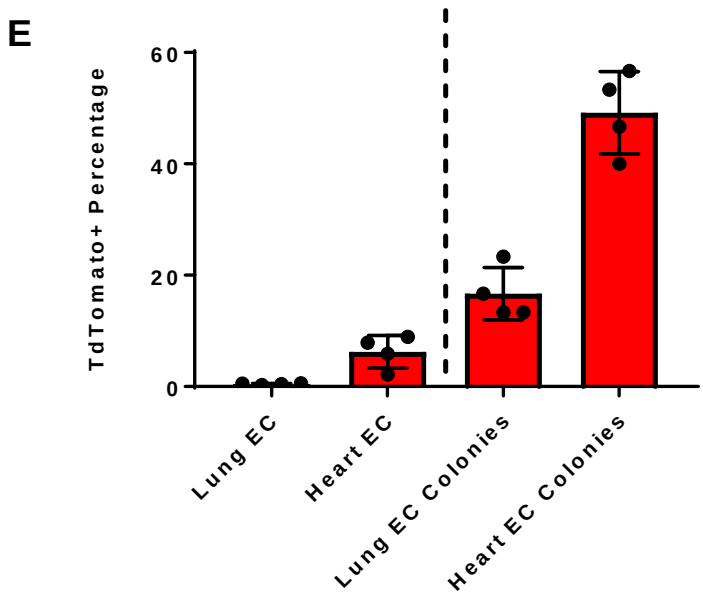
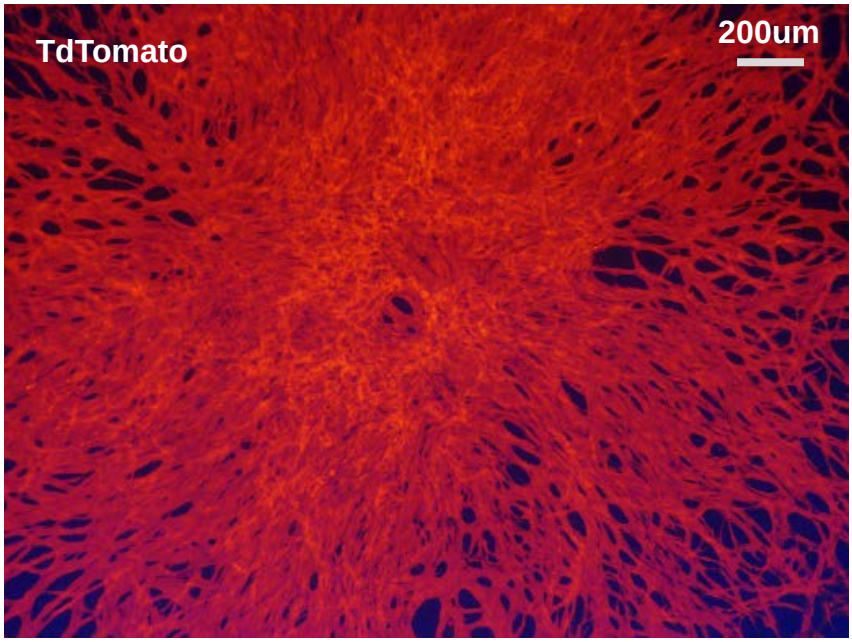


Figure II. 18. *Abcg2*-expressing VESC have *in vitro* EC colony forming potential

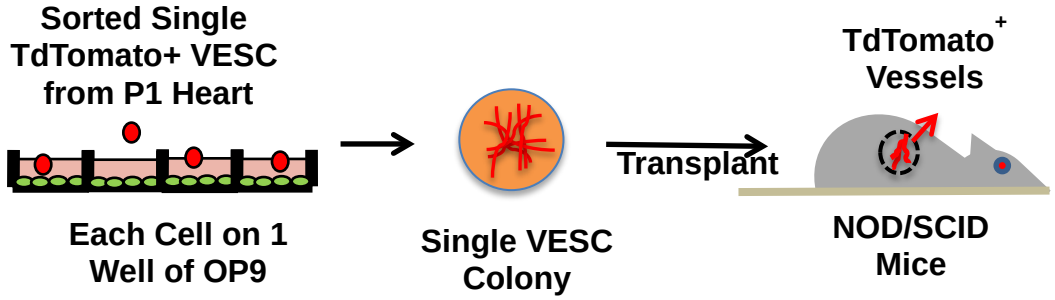
(A). Schematics of *in vitro* colony forming assay and *in vivo* vessel formation assay using *Abcg2*-expressing VESC from ABCG2TT mice. (B). OP9 co-cultured *In vitro* EC colonies (7 days) derived from TdTomato$^+$ (top panels) or TdTomato$^-$ (bottom panels) heart EC of P1 ABCG2TT mice after tamoxifen injection at P0. (C-D) Quantitation of frequencies of colony forming cells in lung (C) and heart (D) TdTomato$^+$ (red bars) and TdTomato$^-$ (blue bars) EC from P1 ABCG2TT mice after tamoxifen injection at P0. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=4 mice from 2 independent experiments). (E). Percentage of TdTomato$^+$ cells in P1 ABCG2TT mice (P0 tamoxifen injected) lung EC, heart EC (determined by flow cytometry) and percentage of TdTomato$^+$ EC colonies in lung EC, heart EC derived colonies. Data represent mean ± s. d. (*n*=4 mice).

Next, we sorted single P1 heart TdTomato$^+$ EC and plated them on OP9 cells to grow single colonies of *Abcg2*-expressing EC derived colonies (Figure II. 19A). After 14 days, we suspended the clonally-derived cells in type 1 collagen gels, and implanted the plugs in the subcutaneous space of host syngeneic mice (Figure II. 19B). After 2 weeks, *Abcg2*-expressing EC derived from each of 6 individual clones gave rise to TdTomato$^+$ vessels *in vivo* (Figure II. 19C), demonstrating their robust clonal *in vivo* blood vessel forming potential.

A


TdTomato
200um

B


Sorted Single
TdTomato+ VESC
from P1 Heart
Each Cell on 1
Well of OP9
Single VESC
Colony
Transplant
TdTomato+
Vessels
NOD/SCID
Mice

C

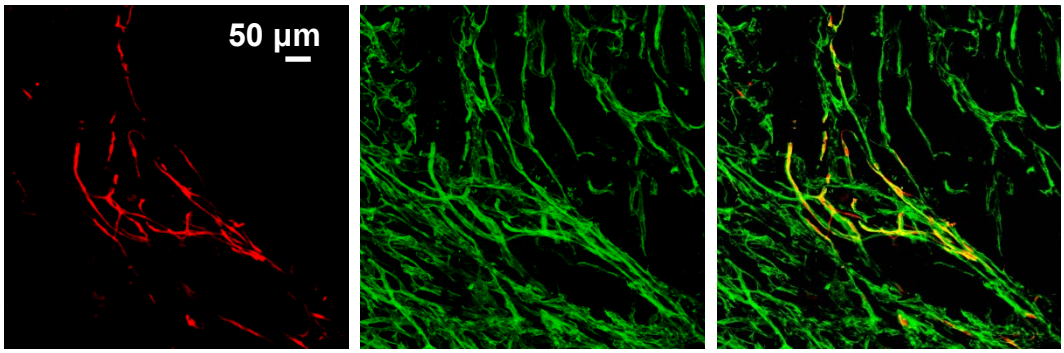
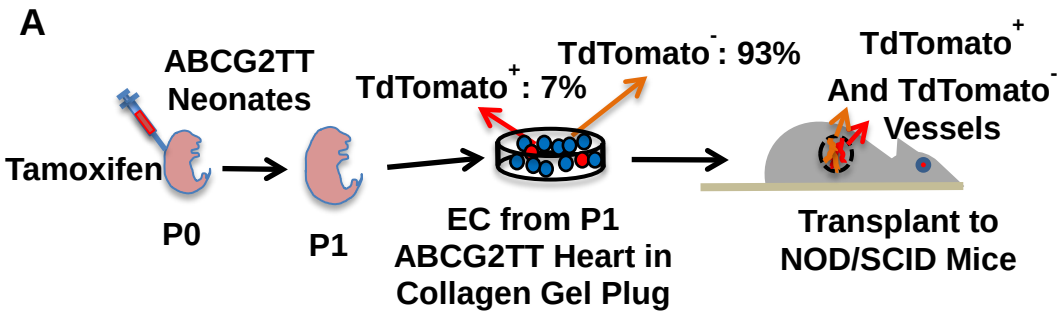
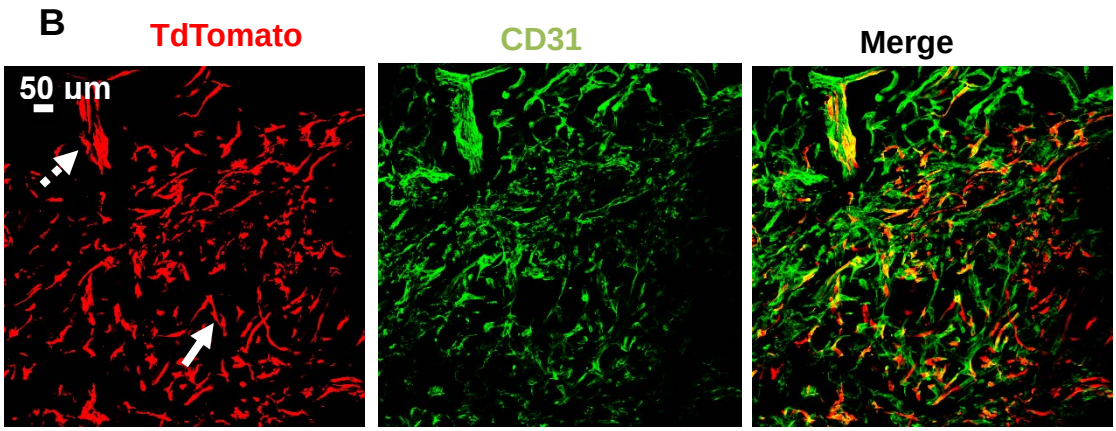

TdTomato
CD31
Merge
50 µm

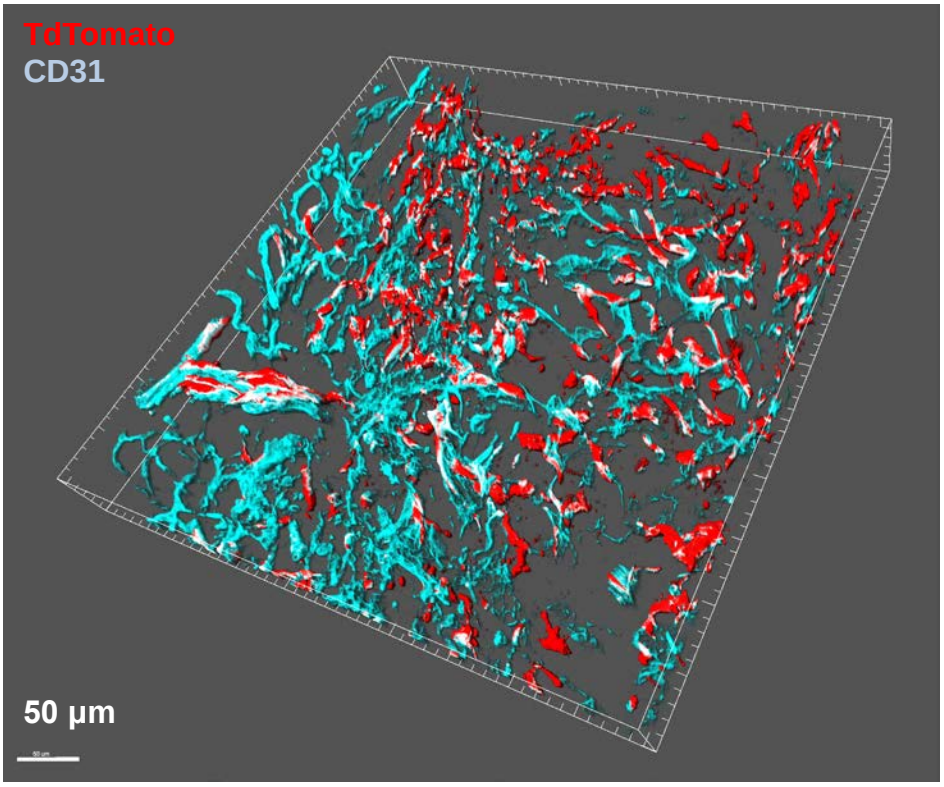
Figure II. 19. A single *Abcg2*-expressing VESC has *in vivo* vessel forming potential. (A). Representative picture of a single P1 ABCG2TT mouse heart TdTomato$^+$ EC derived colony. (B). Schematic of *in vivo* vessel forming potential assay for a single P1 ABCG2TT mouse heart TdTomato$^+$ EC. (C). , Blood vessels formed by a single P1 ABCG2TT mouse heart TdTomato$^+$ EC (Red, TdTomato, Green, CD31). This is a representative picture from 4 experiments using cells from 4 individual mice.

To compare the vessel forming potential of *Abcg2*-expressing TdTomato$^+$ EC with mature TdTomato$^-$ EC, we collected EC from P1 heart tissue and transplanted these cells in collagen gel plugs into recipient mice (Figure II. 20A) at a ratio of 1 TdTomato$^+$ EC for every 9 or 11 TdTomato$^-$ EC (2 studies, Figure II. 20D). Two weeks later, robust donor *Abcg2*-expressing EC derived TdTomato$^+$ vessels were formed in all implanted gels (Figure II. 20B). Those TdTomato$^+$ vessels were primarily capillaries but also contributed to the macrovasculature (>50 microns, Figure II. 20B) in every gel examined (n=3). Importantly, in the retrieved gels, TdTomato$^+$ vessels represented 42.4±2.7% of the total vessel volume (n=3 mice, Figure II. 20C, D), although only 6.7±1.6% of the input EC were TdTomato$^+$ (n=3 mice each of 2 studies, Figure II. 20E). Thus, the *in vivo* vessel forming potential of TdTomato$^+$ *Abcg2*-expressing VESC was 10.8±3.2 fold higher than TdTomato$^-$ EC (Fig. Figure II. 20F).

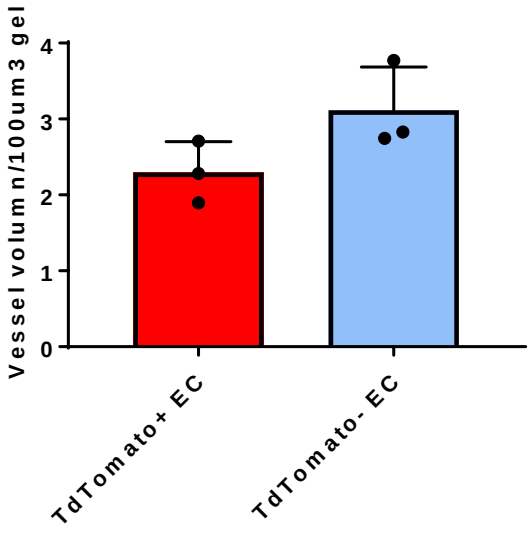

A
ABCG2TT
Neonates
Tamoxifen
P0
P1
TdTomato+: 7%
TdTomato-: 93%
EC from P1
ABCG2TT Heart in
Collagen Gel Plug
TdTomato+
And TdTomato-
Vessels
Transplant to
NOD/SCID Mice


B
TdTomato
CD31
Merge
50 um

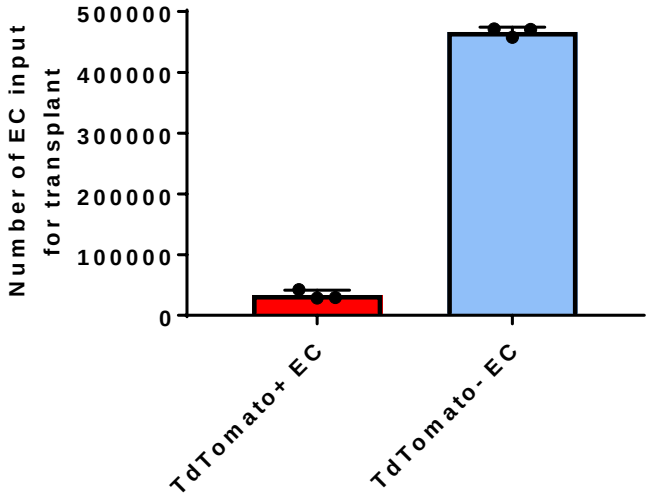
C


TdTomato
CD31
50 µm

D


Vessel volumn/100um3 gel
0
1
2
3
4
TdTomato+ EC
TdTomato- EC

E


Number of EC input for transplant
500000
400000
300000
200000
100000
0
TdTomato+ EC
TdTomato- EC

F

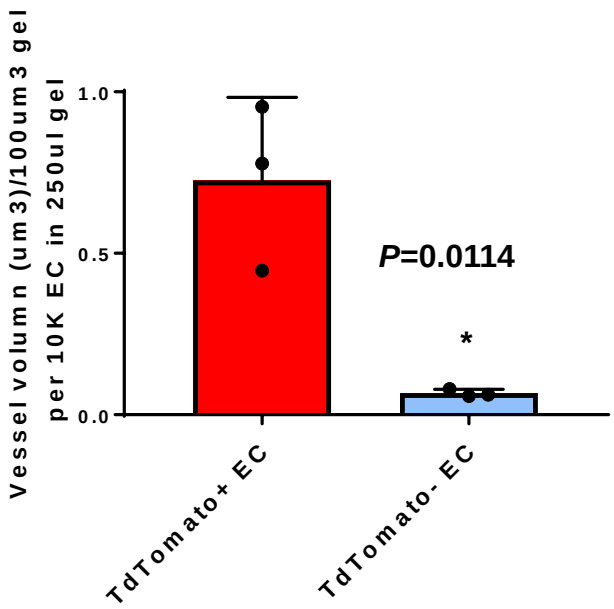
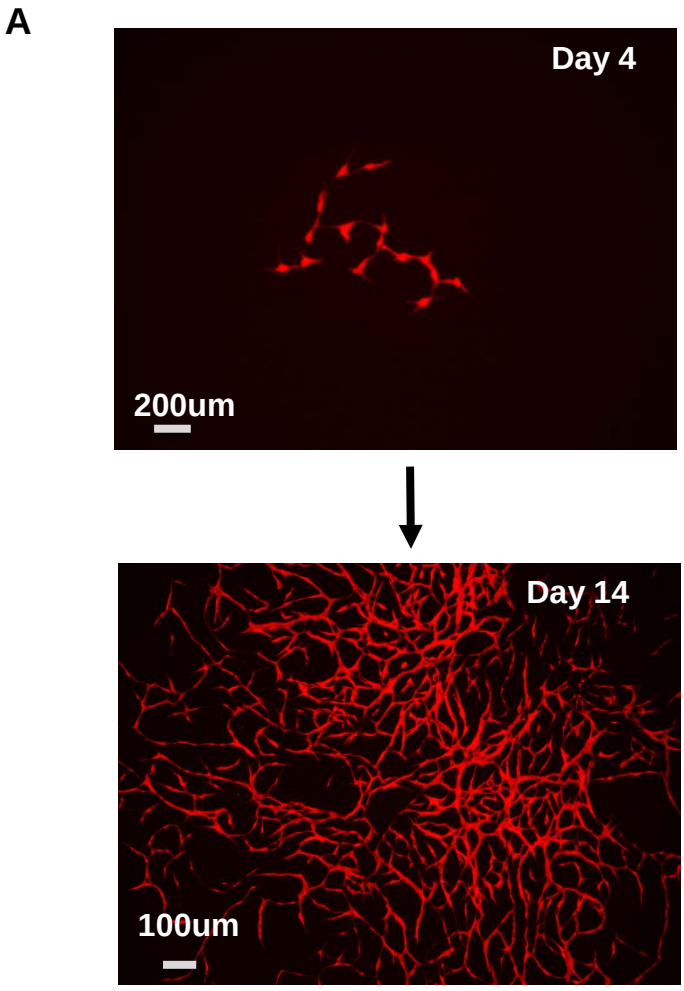

Vessel volumn (um3)/100um3 gel per 10K EC in 250ul gel
1.0
0.5
0.0
P=0.0114
*
TdTomato+ EC
TdTomato- EC

Figure II. 20. *Abcg2*-expressing VESC have *in vivo* vessel forming potential. (A). Schematics of *in vivo* vessel formation assay using *Abcg2*-expressing VESC from ABCG2TT mice. (B). Representative pictures of P1 ABCG2TT heart EC (P0 tamoxifen injected) derived vessels 2 weeks after collagen gel plug transplantation (TdTomato, red, CD31,). This picture is a representative of 3 experiments using 3 individual mice. (C). 3D re-construction of blood vessel formed by P1 ABCG2TT mice (tamoxifen injection at P0) heart EC by Imaris software. Red, Tdtomato, Cyan, CD31. (D). Quantitation of the average volume of $TdTomato^+$ (red bar) and $TdTomato^-$ (blue bar) blood vessels in every $100\mu m^3$ gel. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=3 mice). (E). Numbers of $TdTomato^+$ (red bar) and $TdTomato^-$ (blue bar) EC from P1 ABCG2TT mice (tamoxifen injection at P0) heart EC collagen gel plug transplantation. Data represent mean ± s. d. (*n*=3 mice). (F). Comparison of vessel forming potential of $TdTomato^+$ (red bars) and $TdTomato^-$ (blue bars) EC from P1 ABCG2TT mice after tamoxifen injection at P0. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test (*n*=3 mice).

Additionally, after the gels were digested and re-plated on OP9 stromal cells, TdTomato$^+$ EC from the primary vessels formed secondary colonies that could form secondary vessels in subcutaneous implants of host mice (*n*=6, Figure II. 21). These data indicate that *Abcg2*-expressing EC in developing mice represent VESC that display clonal proliferative potential *in vitro* and *in vivo*, possess greater vasculogenic potential than mature EC not expressing *Abcg2*, give rise to capillary and macrovasculature components that inosculate with the host, and display self-renewal potential in giving rise to primary and secondary blood vasculature *in vivo*.


A
Day 4
200um
Day 14
100um

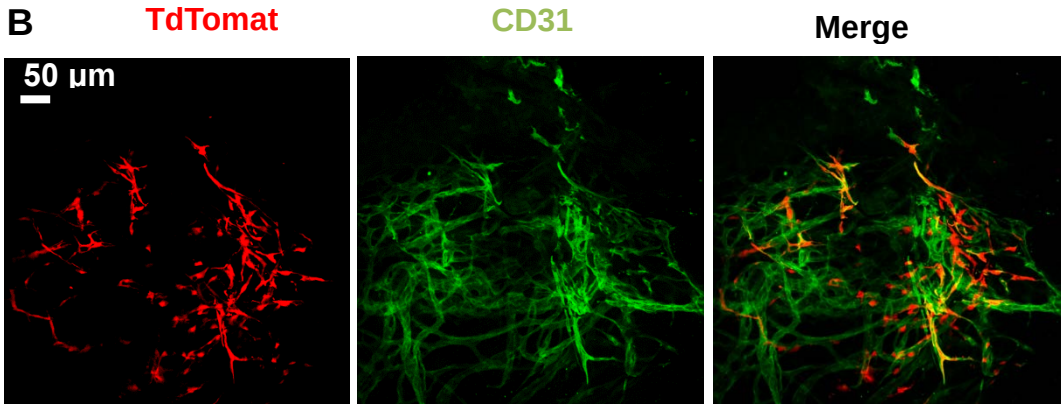
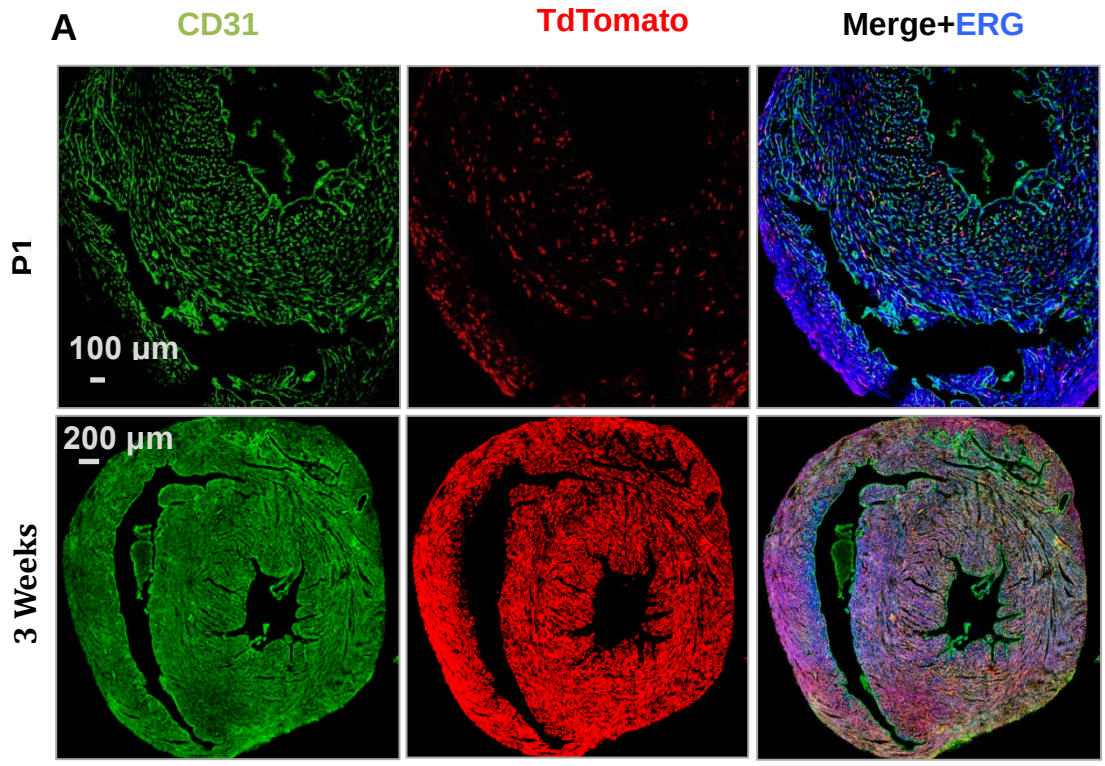

B
TdTomat
CD31
Merge
50 µm

Figure II. 21. *Abcg2*-expressing VESC can self-renew in *in vivo* (A). Representative pictures of a secondary TdTomato$^+$ VESC colony derived from P1 ABCG2TT mice heart EC transplanted gel. (B). Representative pictures of P1 ABCG2TT mice heart TdTomato$^+$ VESC secondary colony EC formed blood vessels after secondary transplantation (represents gels derived from 3 individual mice).

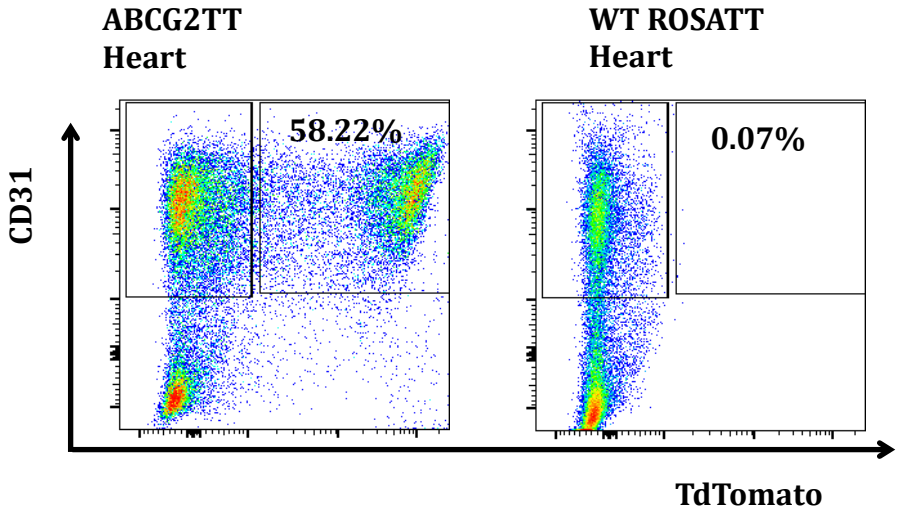
Abcg2-expressing VESC contribute to vessel growth *in vivo* during development

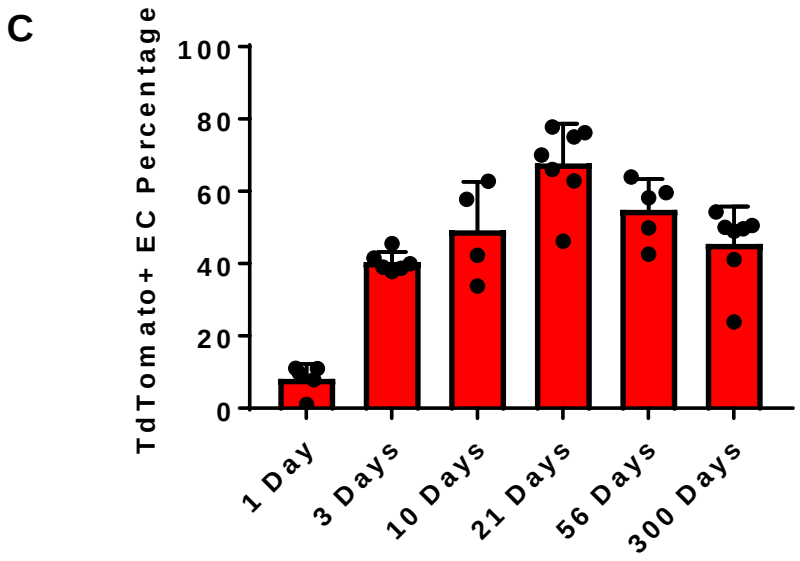
To investigate the contribution of P1 labeled TdTomato$^+$ EC to the growth of blood vessels during postnatal development, 34 ABCG2TT mice that were injected with Tamoxifen at P0 were analyzed at various developmental stages for up to 10 months (Figure II. 22A). Remarkably, *Abcg2*-expressing TdTomato$^+$ EC, which represent only 8.1±4.1% of total EC in the heart at P1 (Figure II. 12, Figure II. 22), contribute to 67.7±11.0% of EC in the heart of 3 week old mice (Fig. 4b, 4d, 4e), including arterial, venous and capillary EC (Fig. 4c), and marked progeny were sustained for up to 300 days after labeling (45.5±10.3%, n=7, Extended Data Fig. 5b). Interestingly, no endocardial cells were found to be derived from TdTomato$^+$ VESC (Extended Data Fig. 5a).

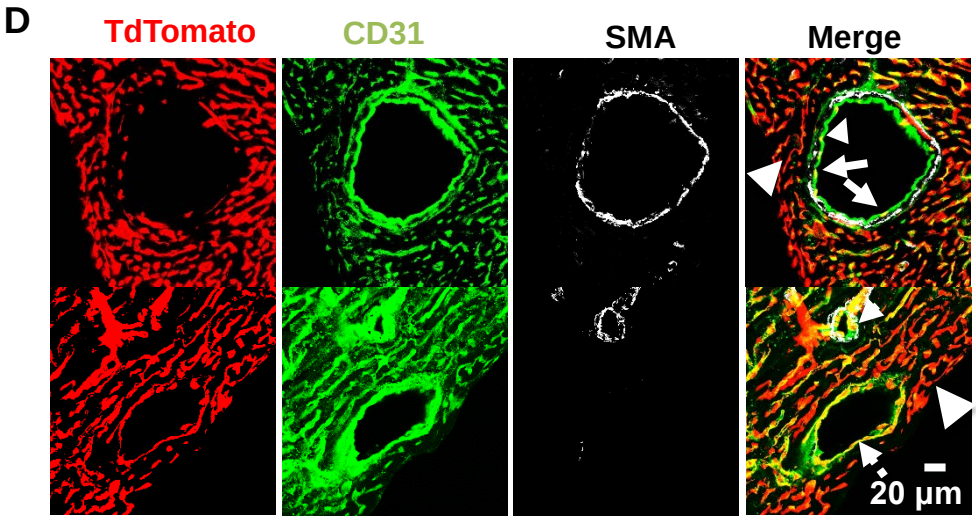


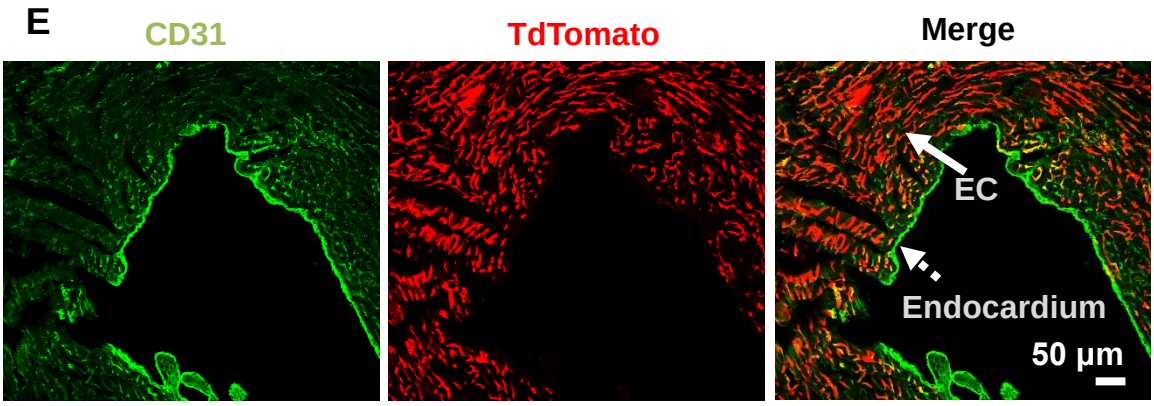
B

From PI$^-$CD45$^-$Ter119$^-$:




C
TdTomato+ EC Percentage
100
80
60
40
20
0
1 Day
3 Days
10 Days
21 Days
56 Days
300 Days


D
TdTomato
CD31
SMA
Merge
20 µm


E
CD31
TdTomato
Merge
EC
Endocardium
50 μm

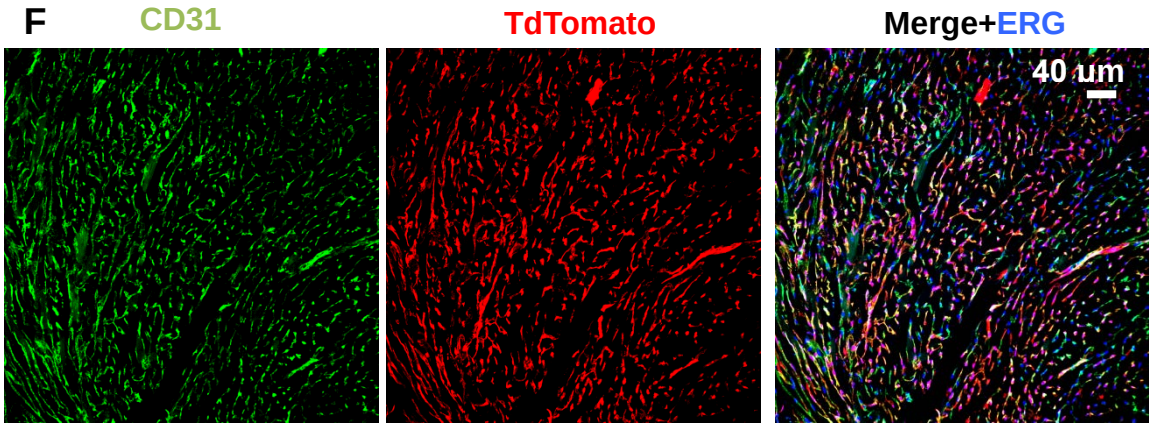
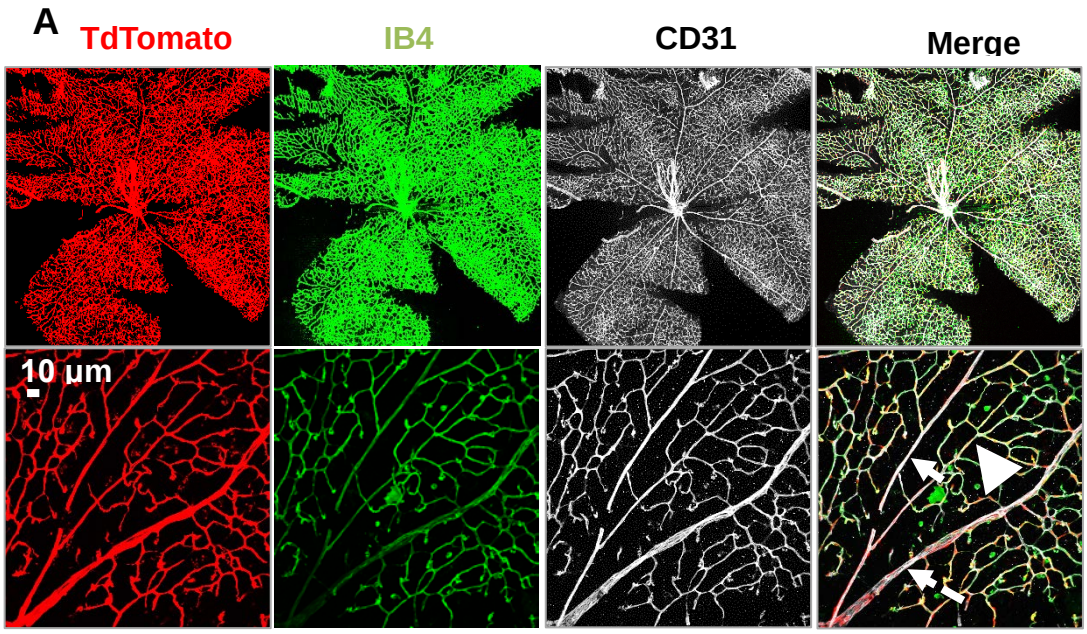

F
CD31
TdTomato
Merge+ERG
40 μm

Figure II. 22. *Abcg2*-expressing VESC contribute to heart vessel growth in vivo during development. (A). Schematics of lineage tracing experiment using ABCG2TT mice to test the contribution of P1 *Abcg2*-expressing VESC to vasculature development in multiple organs. (B). Representative flow cytometry data show the percentage of TdTomato$^+$ cells in 3 week old heart EC of ABCG2TT (left panel) and ROSATdTomato (right panel, WT ROSATT) mice that had received tamoxifen injection at P0. (C). Quantitation of percentage of TdTomato$^+$ EC in multiple developmental stages of heart of ABCG2TT mice after tamoxifen injection at P0. Data represent mean ± s. d. (P0: *n*=5; P3: *n*=6; P10: *n*=4; P21: *n*=7; P56: *n*=5; P300: *n*=7. From 2 independent experiments). (D). Representative pictures show the contribution of P0 labeled TdTomato$^+$ *Abcg2*-expressing VESC to arterial (arrows), venous (dashed arrows) and capillary (arrowheads) of 3 week old ABCG2TT mice heart. Green, CD31; Red, TdTomato; Gray, smooth muscle actin α (SMA). Data represent the results derived from 7 mice. (E). **.** Representative pictures show the contribution of TdTomato$^+$ *Abcg2*-expressing VESC to endothelial cells (EC, arrow) but not endocardium (dashed arrow) of 3 week old ABCG2TT mice heart. Green, CD31; Red, TdTomato. (F). Contribution of TdTomato$^+$ *Abcg2*-expressing VESC to EC of 300 days old mice heart. Green, CD31; Red, TdTomato.

In the retina, TdTomato labeled VESC contributed to essentially all arterial, venous and capillary EC at P10 (Figure II. 23A) and were retained at similar levels at the age of 8 weeks old (Figure II. 23B). Similarly, persistent contributions (up to 10 months) from the VESC labeled at P0 were identified in lung (7.0±2.5% at 3 weeks, *n*=7, 2.4±1.1% at 300 days, *n*=7, Figure II. 24A-D), bone (29.6±12.9% at 3 weeks, *n*=7, 17.8±6.5% at 300 days, *n*=7, Figure II. 25A-D), and skeletal muscle (Figure II. 26) residing in arteries, veins, and capillaries (Figure II. 23D, Figure II. 24D, Figure II. 25D), although the degree of contribution varied among the organs. These results demonstrate that *Abcg2*-expressing VESC contribute long term to mature progeny in the vascular system (arteries, veins, capillaries) in multiple tissues during normal murine growth and development.


A
TdTomato
IB4
CD31
Merge
10 µm

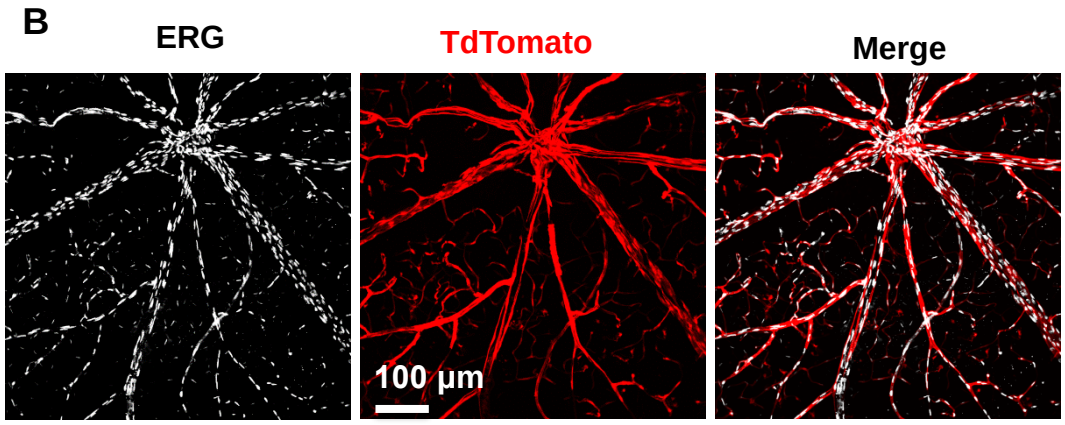
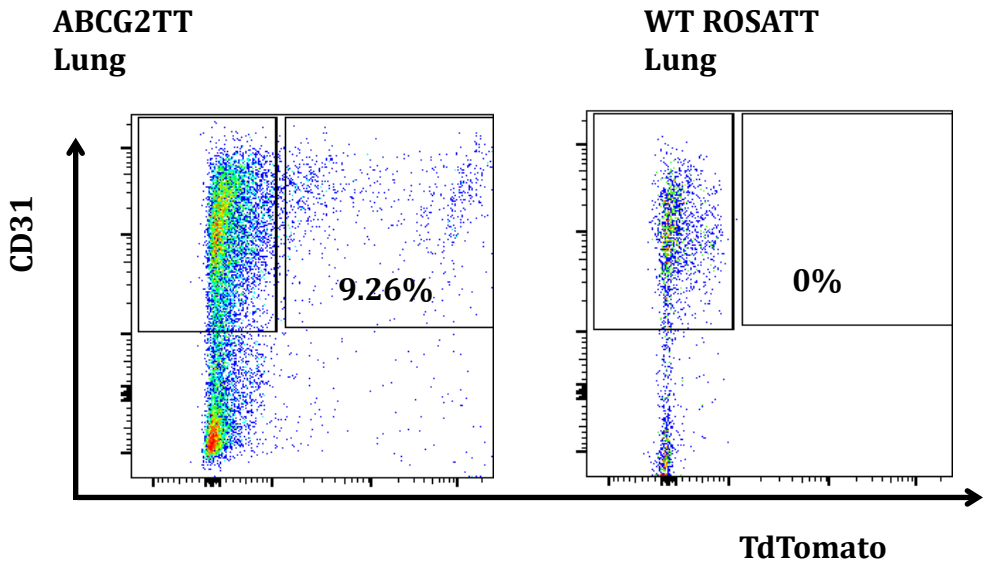

B
ERG
TdTomato
Merge
100 µm

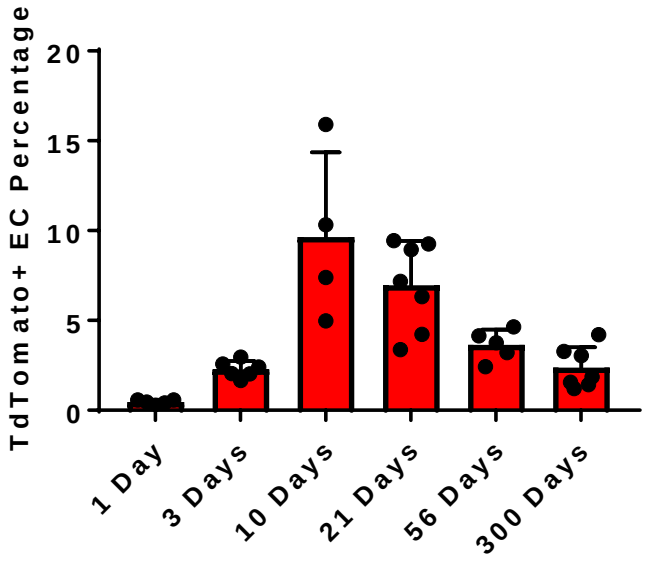
Figure II. 23. *Abcg2*-expressing VESC contribute to retinal vessel growth *in vivo* during development. (A). Contribution of P0 labeled TdTomato$^+$ *Abcg2*-expressing VESC to 10 days old ABCG2TT mice retinal vasculatures include arteries (arrow), veins (dashed arrow) and capillaries (arrowhead). Red, TdTomato; Green, isolectin B4 (IB4); Gray, CD31. (represents results derived from 4 individual mice). (B). Contribution of TdTomato$^+$ EC to 8 week old mice retinal EC. Gray, ERG; Red, TdTomato. **d,** P1 (top panels) and 3 week old (bottom panels) ABCG2TT mice lung. Green, CD31; Red, TdTomato; blue, ERG.

A

From PI$^-$CD45$^-$Ter119$^-$:



B



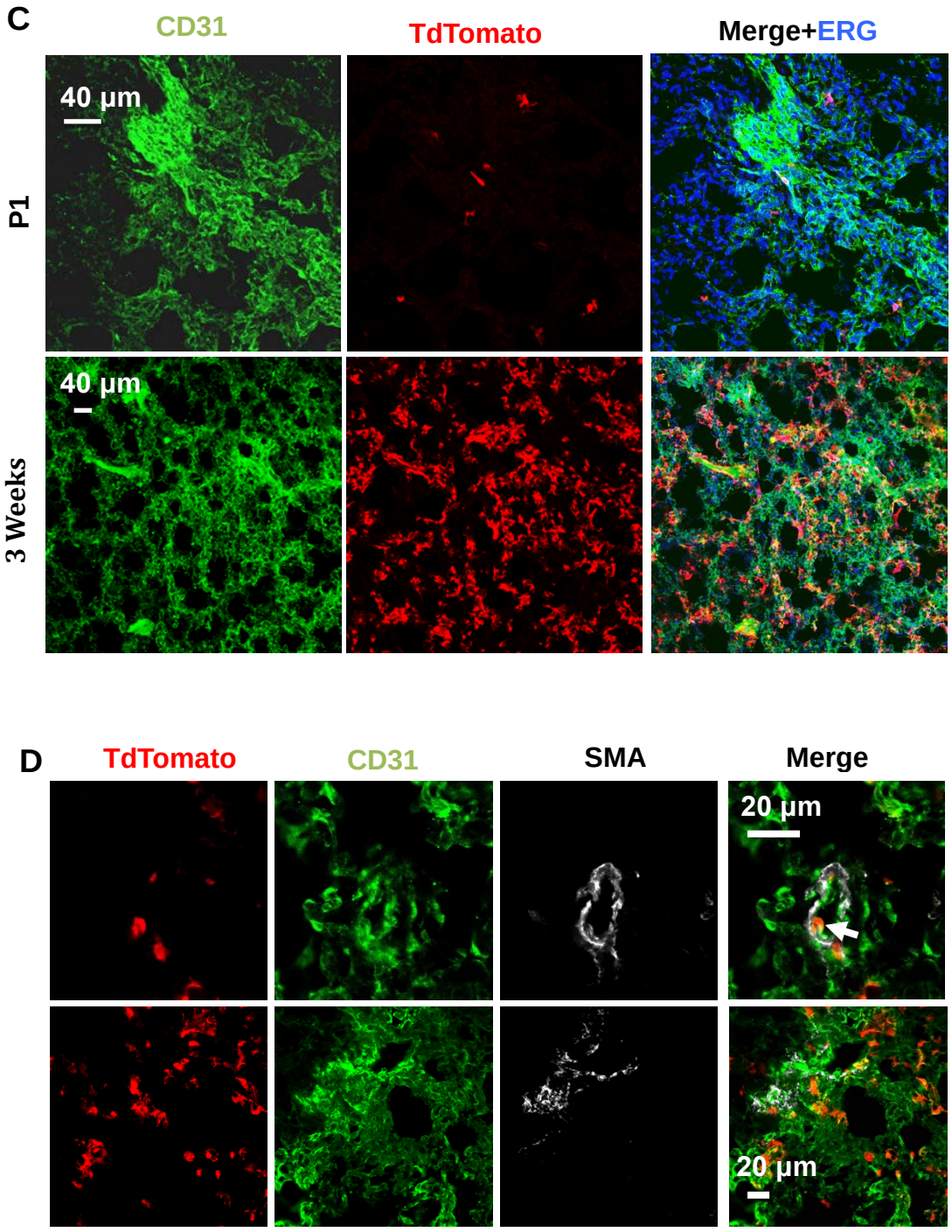
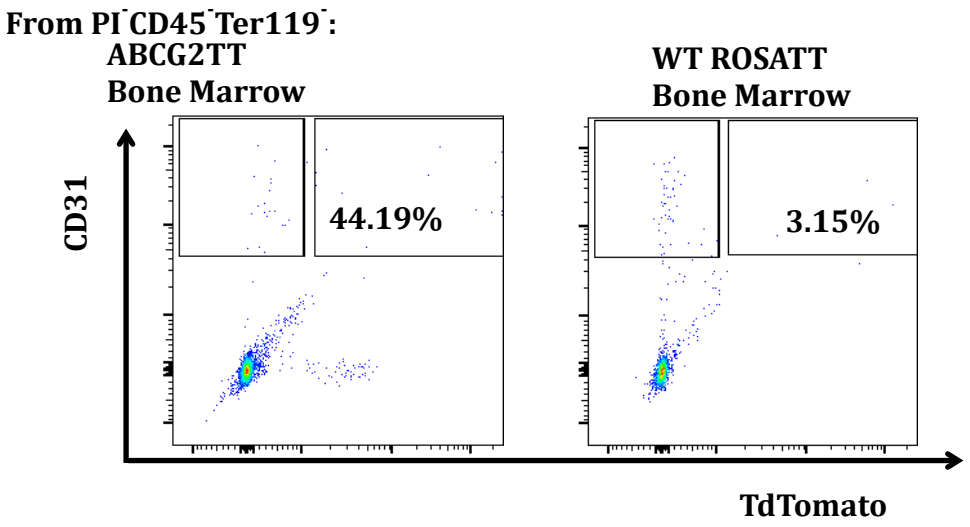
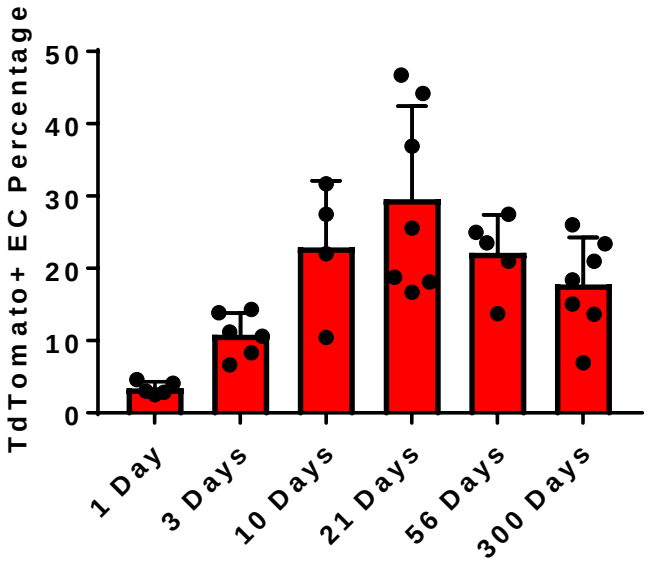

C
CD31
TdTomato
Merge+ERG
P1
3 Weeks
40 µm
40 µm
D
TdTomato
CD31
SMA
Merge
20 µm
20 µm

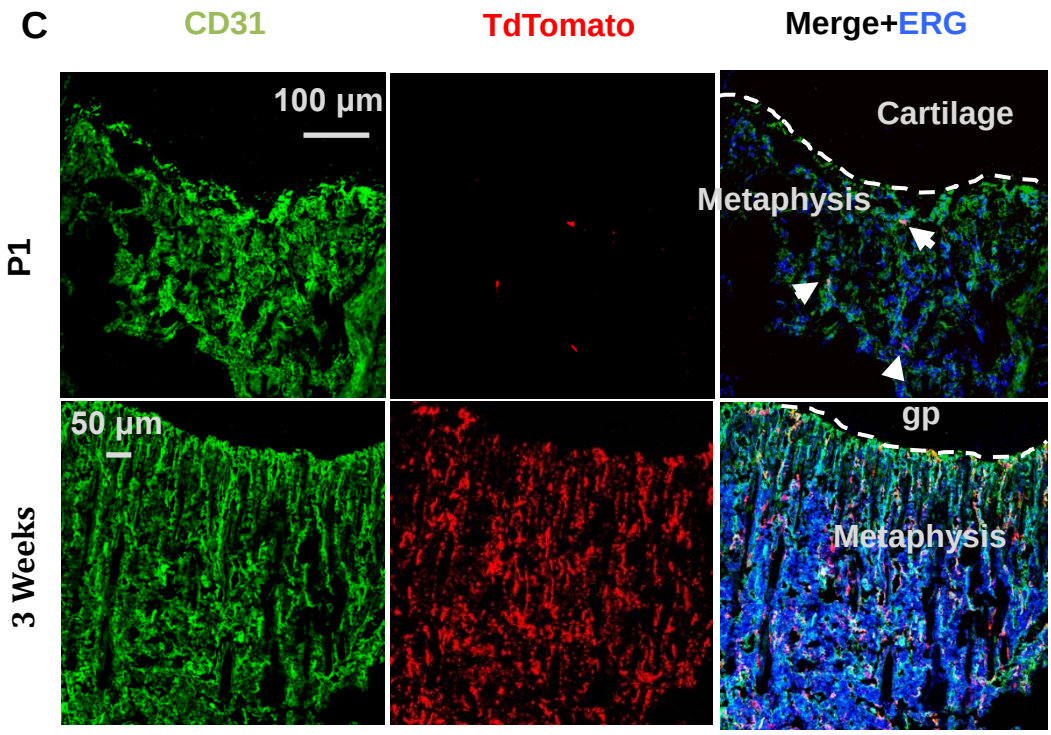
Figure II. 24. *Abcg2*-expressing VESC contribute to lung vessel growth *in vivo* during development. (A). , Flow cytometry data show the percentage of TdTomato$^+$ cells in 3 week old lung EC of ABCG2TT (left panels) and ROSATdTomato (right panels, WT ROSATT) mice that had received tamoxifen injection at P0. (B). Quantitation of percentage of TdTomato$^+$ EC in multiple developmental stages of lung of ABCG2TT mice after tamoxifen injection at P0. Data represent mean ± s. d. (P0: *n*=5; P3: *n*=6; P10: *n*=4; P21: *n*=7; P56: *n*=5; P300: *n*=7. From 2 independent experiments). (C). P1 (top panels) and 3 week old (bottom panels) ABCG2TT mice lung. Green, CD31; Red, TdTomato; blue, ERG. (D). Contribution of TdTomato$^+$ EC to 3 week old lung arterial EC (top panels, arrow) and capillary EC (bottom panels). Green, CD31; Red, TdTomato; Gray, smooth muscle actin α (SMA).

A


From PI-CD45-Ter119-:
ABCG2TT
Bone Marrow
WT ROSATT
Bone Marrow
CD31
44.19%
3.15%
TdTomato

B


TdTomato+ EC Percentage
0
10
20
30
40
50
1 Day
3 Days
10 Days
21 Days
56 Days
300 Days


C
CD31
TdTomato
Merge+ERG
P1
3 Weeks
100 µm
50 µm
Cartilage
Metaphysis
gp
Metaphysis

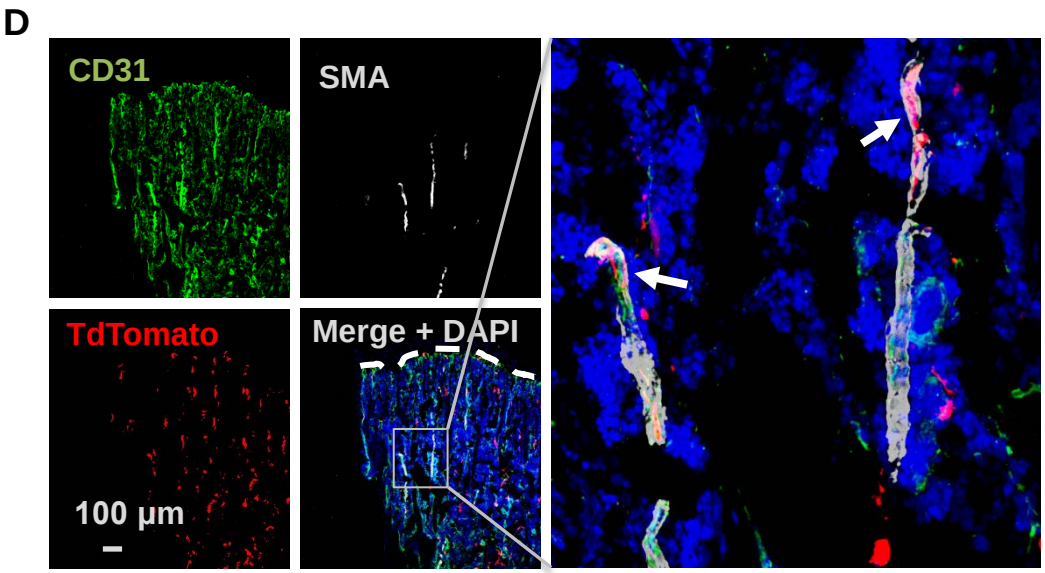

D
CD31
SMA
TdTomato
Merge + DAPI
100 µm

Figure II. 25. *Abcg2*-expressing VESC contribute to bone marrow vessel growth *in vivo* during development. (A). Flow cytometry data show the percentage of TdTomato$^+$ cells in 3 week old bone marrow EC of ABCG2TT (left panels) and ROSATdTomato (right panels, WT ROSATT) mice that had received tamoxifen injection at P0. (B). Quantitation of percentage of TdTomato$^+$ EC in multiple developmental stages of bone marrow of ABCG2TT mice after tamoxifen injection at P0. Data represent mean ± s. d. (P0: *n*=5; P3: *n*=6; P10: *n*=4; P21: *n*=7; P56: *n*=5; P300: *n*=7. From 2 independent experiments). (C). P1 (top panels) and 3 week old (bottom panels) ABCG2TT mice bone marrow. Green, CD31; Red, TdTomato; blue, ERG. (D). Contribution of TdTomato$^+$ EC to 3 week old tibia capillaries and arteries (arrows). Green, CD31; Red, TdTomato; Gray, smooth muscle actin α (SMA).

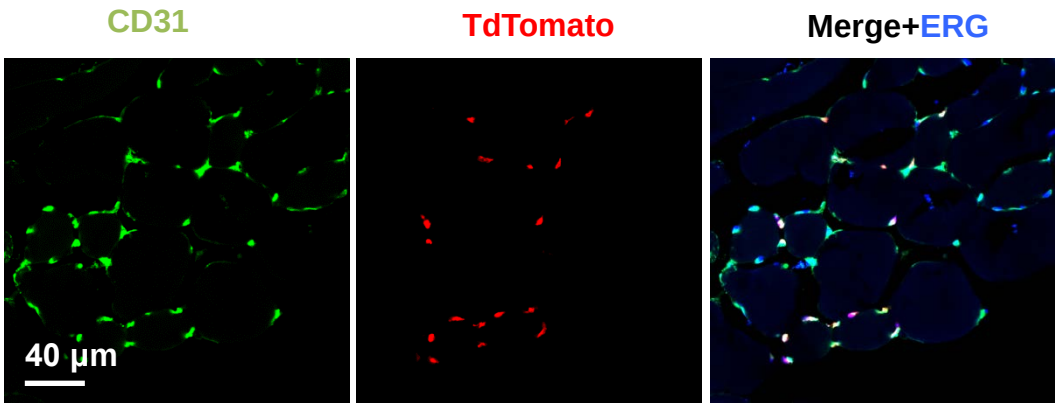
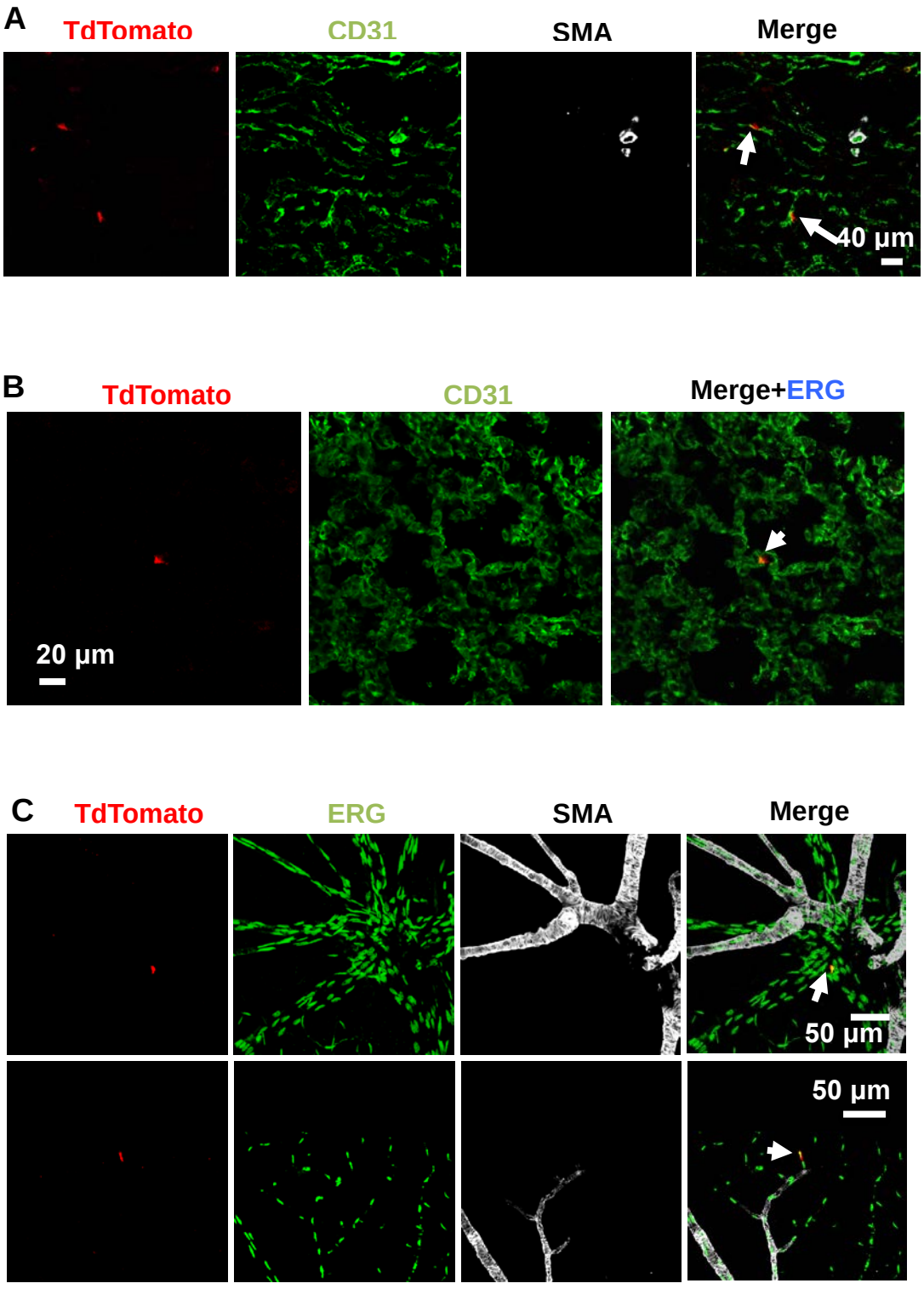


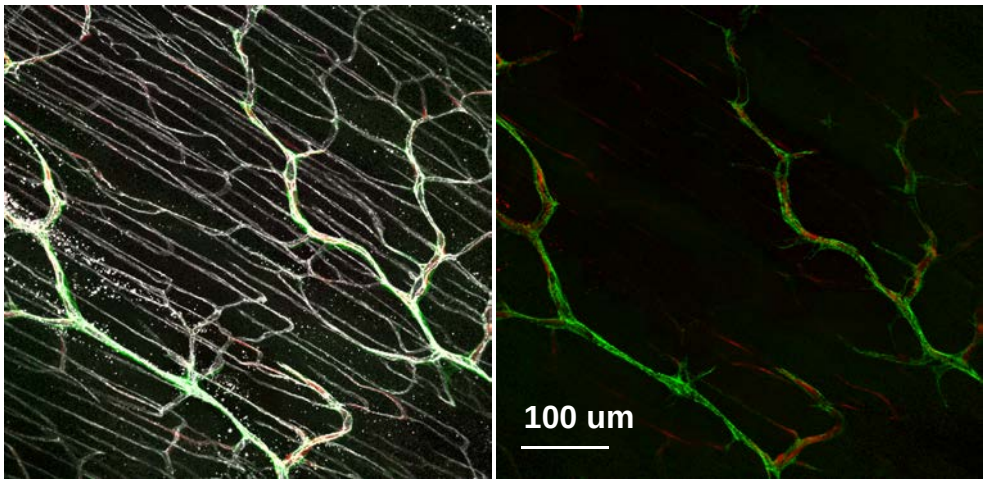
Figure II. 26. *Abcg2*-expressing VESC contribute to skeletal muscle vessel growth *in vivo* during development. Contribution of TdTomato⁺ *Abcg2*-expressing VESC to EC of 300 days old mice skeletal muscle. Green, CD31; Red, TdTomato; blue, ERG.

Abcg2-expressing VESC are maintained in adult blood vessels

Previous studies using the same *Abcg2CreERT* mice have shown that EC labeled by Tamoxifen activation in adult mice led to robust contribution to vessel growth and regeneration after cardiac and skeletal muscle injury (Doyle et al., 2011; Maher et al., 2014), though the cells that gave rise to these labeled progeny were not identified (Doyle et al., 2011; Maher et al., 2014). This suggests that *Abcg2*-expressing VESC reside and are active in adult tissue vascular beds challenged with injury. Indeed, when a single dose of tamoxifen was administered into 6 week old ABCG2TT mice, single TdTomato$^+$ EC could also be identified in multiple tissues include heart (Figure II. 27A), lung (Figure II. 27B), retina (Figure II. 27C), skeletal muscle (Figure II. 27D) and kidney (Figure II. 27E) after 24 hours, but their frequency was significantly decreased compared to the P1 mice (Figure II. 28) and the change in frequency was correlated with decreased total ECFC potential in adult tissue EC (Figure II. 29).


A
TdTomato
CD31
SMA
Merge
40 μm
B
TdTomato
CD31
Merge+ERG
20 μm
C
TdTomato
ERG
SMA
Merge
50 μm
50 μm

D



E

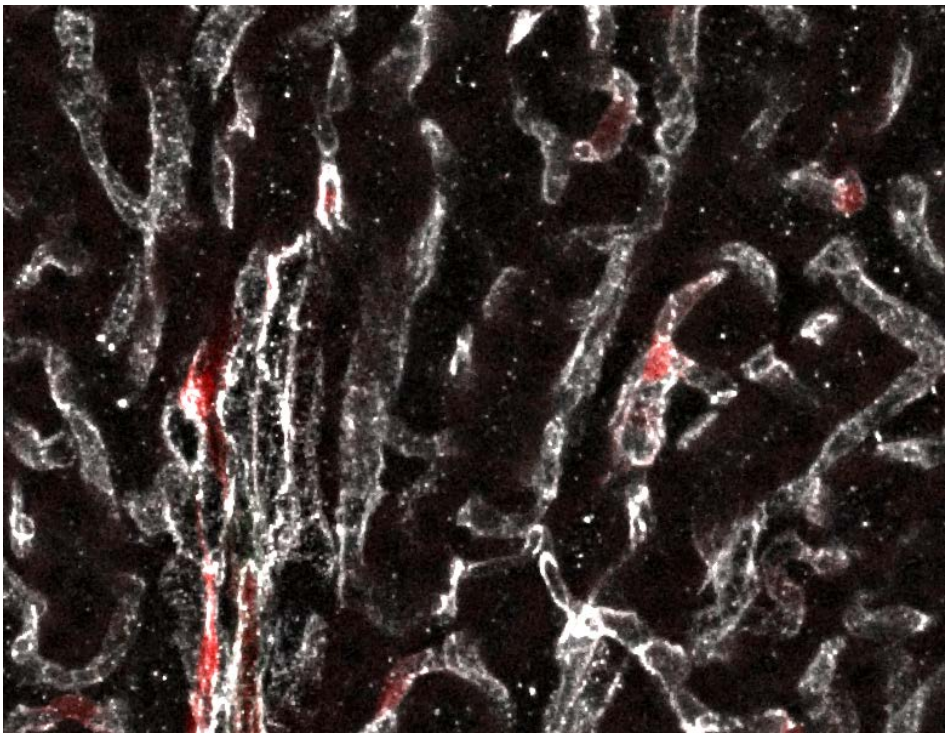


Figure II. 27. *Abcg2*-expressing VESC exist in adult mice tissues. (A). Representative pictures show the distribution of TdTomato$^+$ VESC (arrows) in 6 week old heart of ABCG2TT mice 24 hours after tamoxifen injection. Red, TdTomato; Green, CD31; Gray, smooth muscle actin α (SMA). (B). TdTomato$^+$ EC in lung (arrow). Green, CD31; Red, TdTomato. (C). TdTomato$^+$ *Abcg2*-expressing EC in vein (top panels, arrow) and capillaries (bottom panel, arrow) of retina. Green, CD31; Red, TdTomato; Gray, smooth muscle actin α (SMA). (D). TdTomato$^+$ EC in skeletal muscle (arrow). Green, smooth muscle actin α; Gray, CD31; Red, TdTomato. (E). TdTomato$^+$ EC in kidney. Gray, CD31; Red, TdTomato.

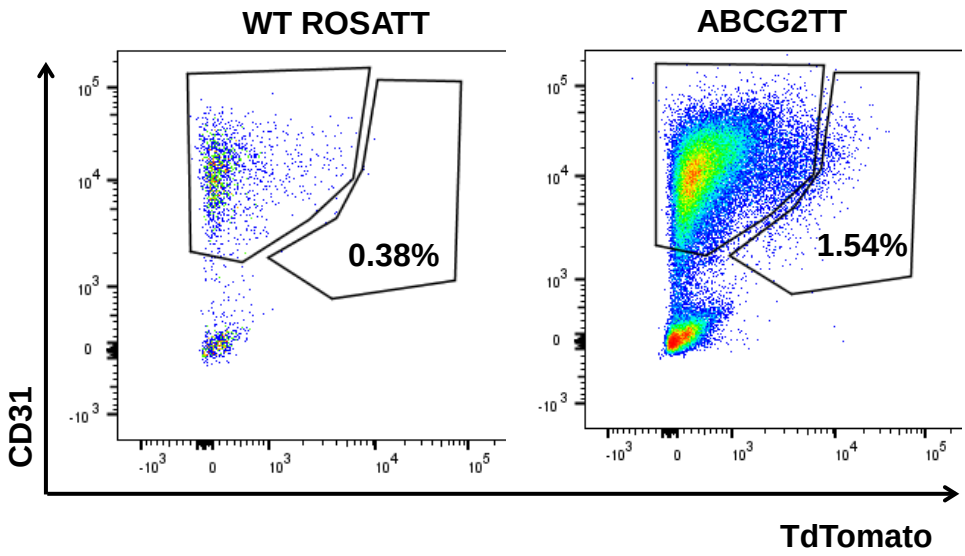
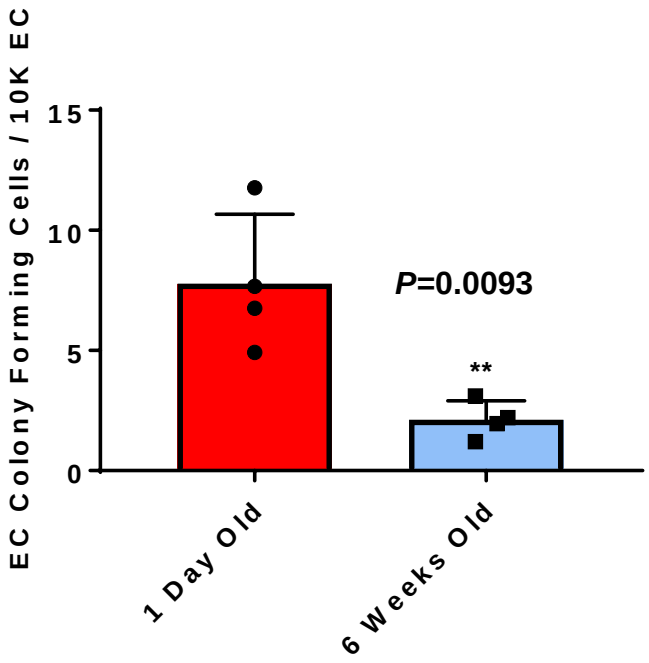


Figure II. 28. Flow cytometry data show the percentage of TdTomato⁺ cells in 6 week old heart EC of ABCG2TT (right panel) and ROSATdTomato (left panel, WT ROSATT) mice that had received tamoxifen injection 24 hours before the experiment.

A


EC Colony Forming Cells / 10K EC
15
10
5
0
P=0.0093
**
1 Day Old
6 Weeks Old

B

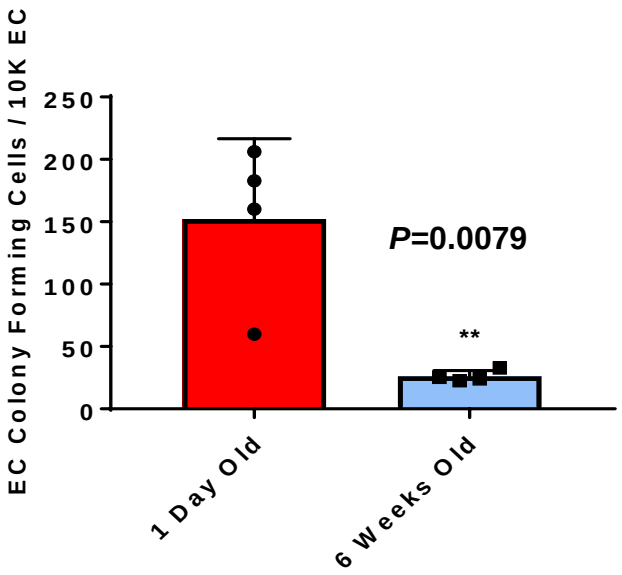
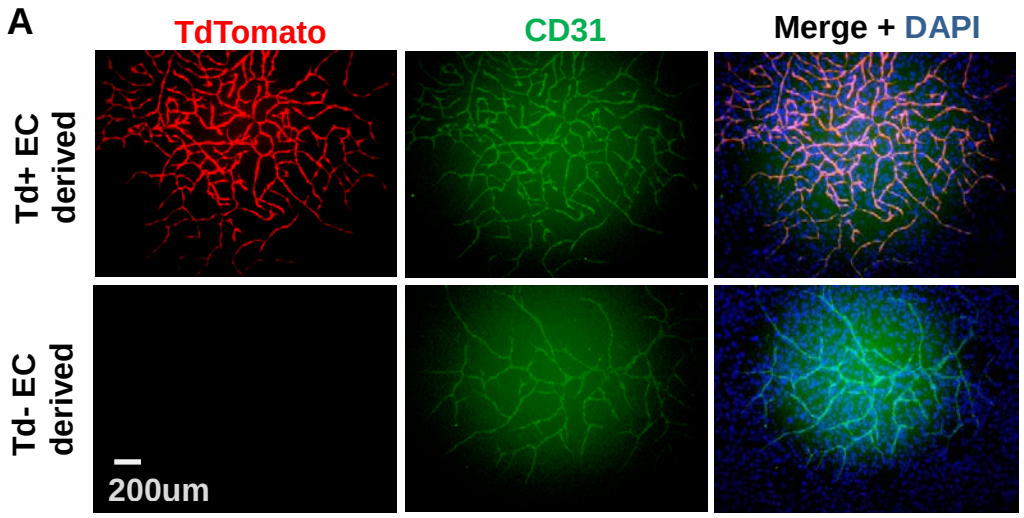

EC Colony Forming Cells / 10K EC
250
200
150
100
50
0
P=0.0079
**
1 Day Old
6 Weeks Old

Figure II. 29. Frequency of colony forming EC in P1 (red bars) and 6 week old (blue bars) heart (A) and lung (B) EC. Data represent mean ± s. d. p values, two-tailed unpaired t-test. (n=4 mice from 2 independent experiments).

Adult tissue *Abcg2*-expressing VESC were also enriched in ECFC potential *in vitro* compared to TdTomato$^-$ EC (Figure II. 30), similar to VESC identified in the neonatal period (Figure II. 18).

To test the potential of adult VESC to contribute to adult vessel regeneration upon injury, we induced experimental hind limb ischemia injury to nude mice and injected heart TdTomato$^+$ EC of 6 week old ABCG2TT mice 24 hours after tamoxifen injection. After 6 weeks, while only 64.5±11.0% of blood flow in injured legs was restored in control mice (Figure II. 31), the blood flow in injured legs of mice that received adult TdTomato$^+$ VESC injections was comparable with uninjured legs (103.00±18.58%, n=10 mice, Figure II. 31A, B). Importantly, robust adult VESC derived TdTomato$^+$ blood vessels could be detected from all mice that received cell injections (Figure II. 31C-E). These TdTomato$^+$ vessels included both capillaries, arterioles and major arteries that were larger than 100μm in diameter (Figure II. 31D, E). Thus, *Abcg2*-expressing VESC with the potential to regenerate capillaries, arterioles, and larger arteries in ischemic tissues are retained in adult tissue vasculature.


A
TdTomato
CD31
Merge + DAPI
Td+ EC derived
Td- EC derived
200um

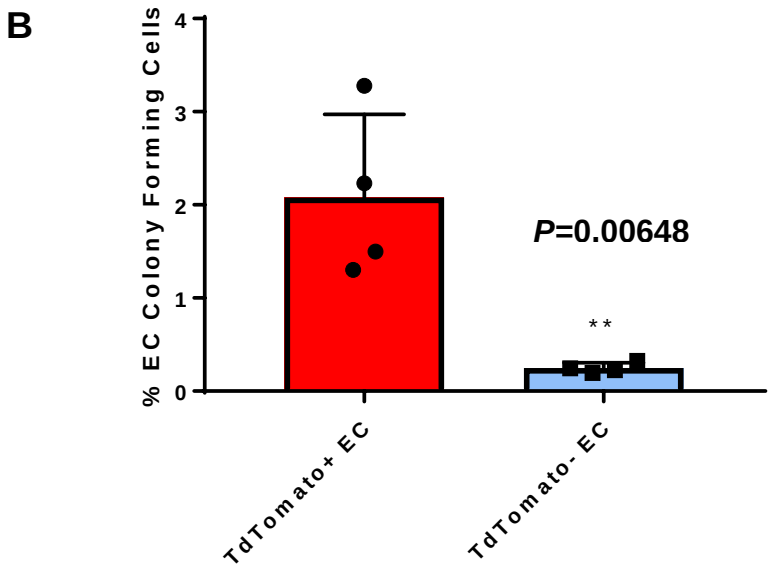
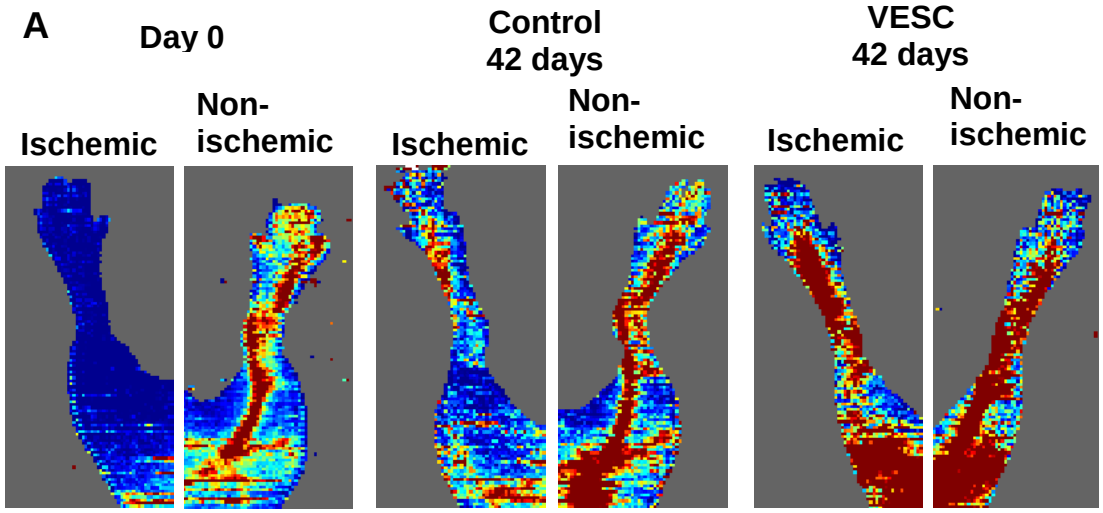
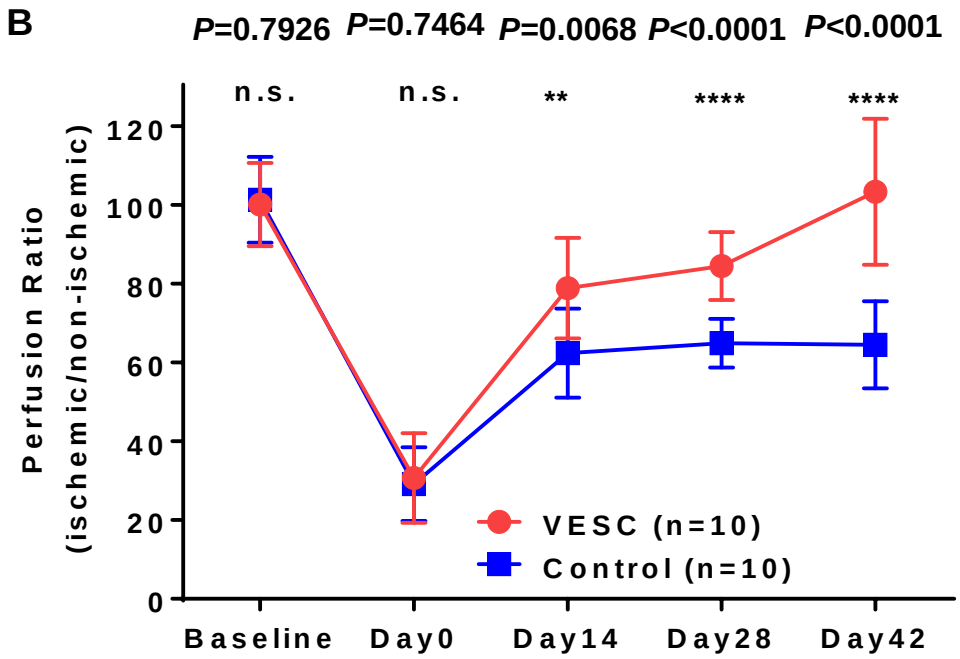
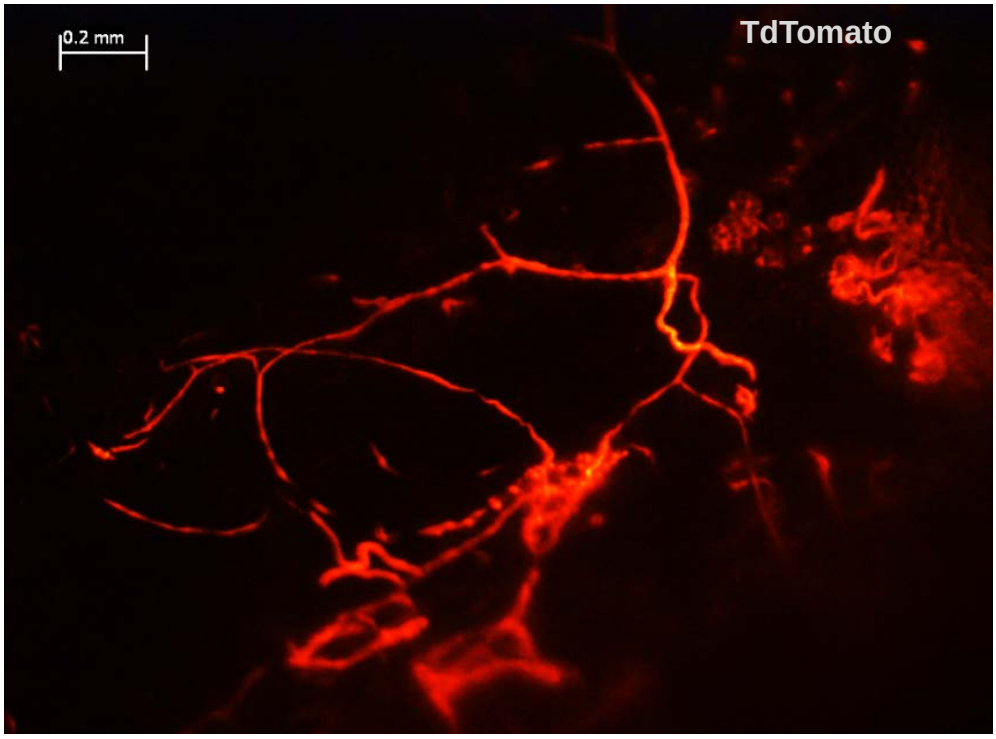

B
% EC Colony Forming Cells
4
3
2
1
0
P=0.00648
**
TdTomato+ EC
TdTomato- EC

Figure II. 30. *Abcg2*-expressing VESC in adult mice tissues have *in vitro* colony forming potential (A). Representative pictures of EC colonies derived from TdTomato^{+} EC (top panels) and TdTomato^{-} EC (bottom panels) from 6 week old ABCG2TT mice heart 24 hours after single tamoxifen injection. (B). Frequency of colony forming cells in TdTomato^{+} and TdTomato^{-} EC from 6 week old ABCG2TT heart with tamoxifen injected 24 hours before sorting. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (*n*=4 mice).

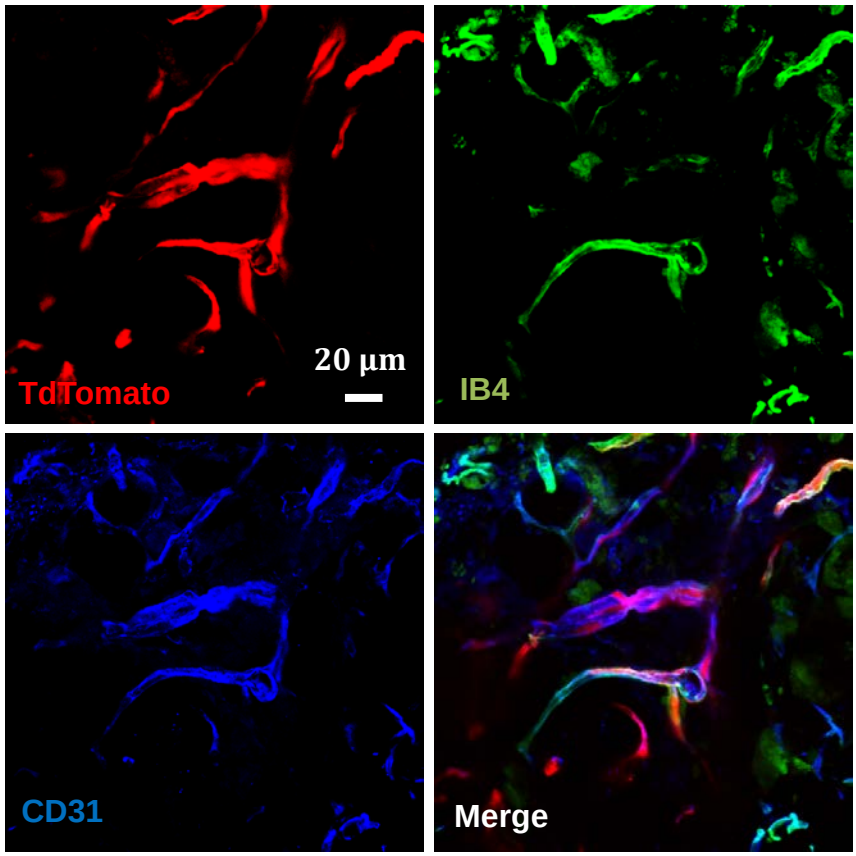

A
Day 0
Control
42 days
VESC
42 days
Ischemic
Non-
ischemic
Ischemic
Non-
ischemic
Ischemic
Non-
ischemic


B
P=0.7926
P=0.7464
P=0.0068
P<0.0001
P<0.0001
n.s.
n.s.
**
****
****
Perfusion Ratio
(ischemic/non-ischemic)
120
100
80
60
40
20
0
VESC (n=10)
Control (n=10)
Baseline
Day0
Day14
Day28
Day42

C



D



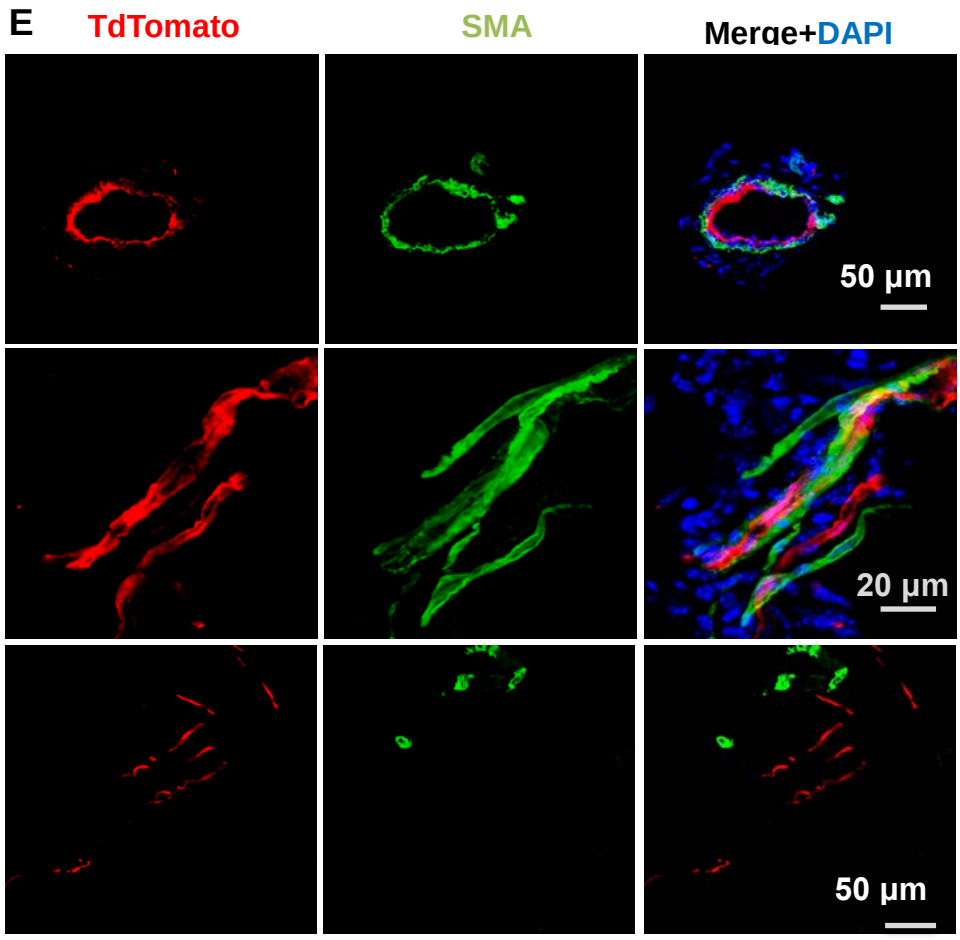
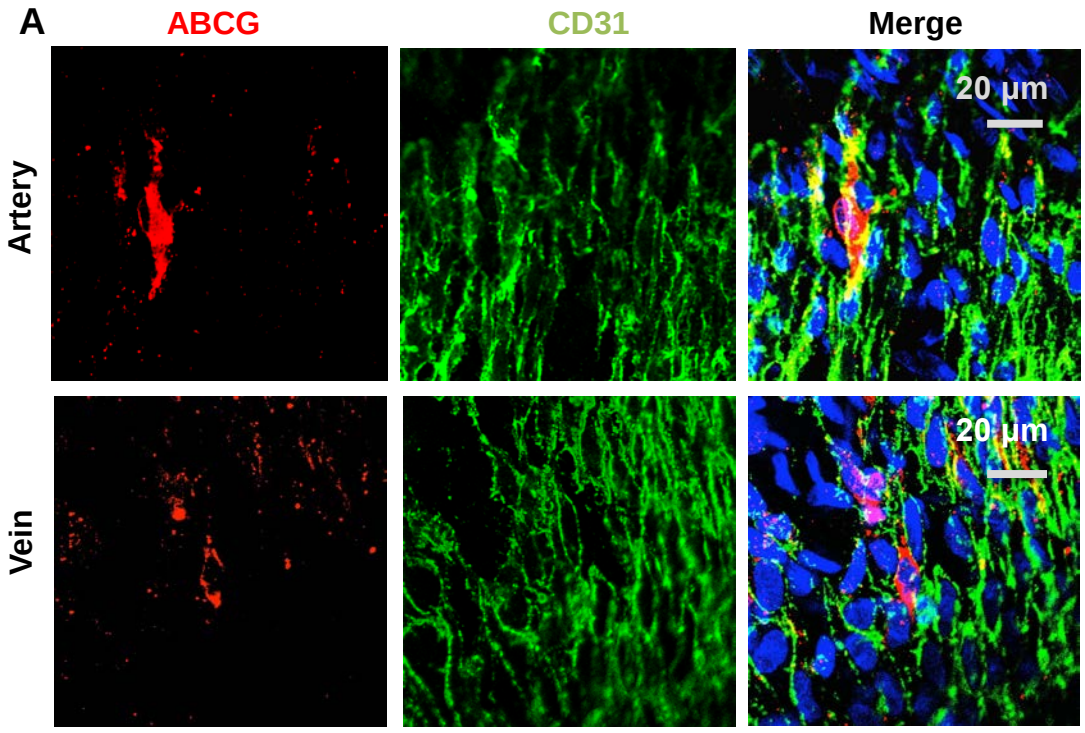

E
TdTomato
SMA
Merge+DAPI
50 μm
20 μm
50 μm

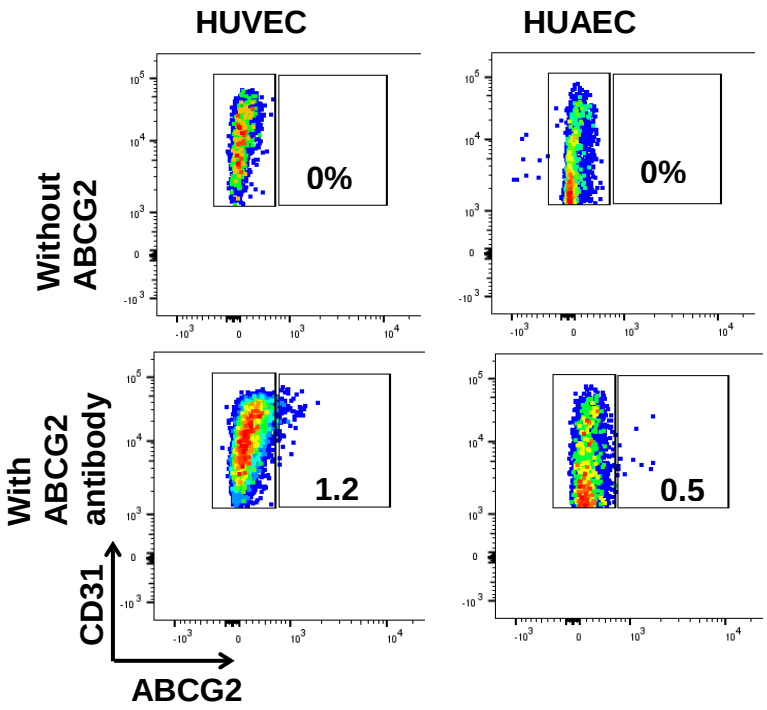
Figure II. 31. *Abcg2*-expressing VESC in adult mice tissues can contribute to vessel regeneration (A). Rescue of blood flow in the legs of nude mice after hind limb ischemia. After surgery, hind limb muscles of nude mice were injected with PBS or 100K TdTomato$^+$ CD31$^+$CD45$^-$ EC from 6 week old ABCG2TT heart that had received tamoxifen injected 24 hours before experiment. Representative pictures of blood flow of ischemia induced legs (left of each panel) and uninjured control legs (right of each panel) of nude mice at day 0 of experiment (left panel) or 42 days after injected with PBS (middle panel) or TdTomato$^+$ EC (right panels). (B). Quantitation of the recovery of blood flow in ischemia legs of nude mice received TdTomato$^+$ VESC (red) or PBS (blue). Blood flow in uninjured legs were used as references. Data represent mean ± s. d. *p* values, two-tailed unpaired t-test. (n=10 mice from 3 independent experiments). (C, D). Contribution of TdTomato$^+$ VESC to recipient blood vessels. Red, TdTomato; Green, isolectin B4 (IB4); Blue, CD31. (E). Contribution of TdTomato$^+$ EC from 6 week old ABCG2TT mice (tamoxifen injected 24 hours before collection) to the arteries (top panels), arterioles (middle panels), and capillaries (bottom panels) of nude mice ischemic leg muscle 42 days after cell injection.

Human VESC are labeled by ABCG2

It has been known for a decade that human umbilical cord artery and vein contain EC with *in vitro* clonal colony forming potential (Ingram et al., 2005). However a marker that labels these colony forming cells prospectively is still lacking. To assess if *ABCG2*-expressing VESC also exist in human umbilical vessels, we labeled freshly isolated human umbilical artery and vein samples with the 5D3 anti-ABCG2 monoclonal antibody (Taylor et al., 2017; Zhou et al., 2001) and performed flow cytometry and immunofluorescent analysis. $ABCG2^+$ EC are readily apparent in human blood vessel EC and represent, 0.5 to 10%, respectively of total human umbilical cord arterial EC (HUAEC) and vein EC (HUVEC) (Figure II. 32). When $ABCG2^+$ cells from freshly isolated HUVEC were sorted by magnetic activated cell sorting (MACS, Figure II. 33A) and cultured on type 1 collagen coated plates, $ABCG2^+$ HUVEC showed significantly superior ECFC potential than $ABCG2^-$ HUVEC (Figure II. 33B, C).


A
ABCG
CD31
Merge
Artery
Vein
20 μm
20 μm

B From PI$^-$CD45$^-$



C

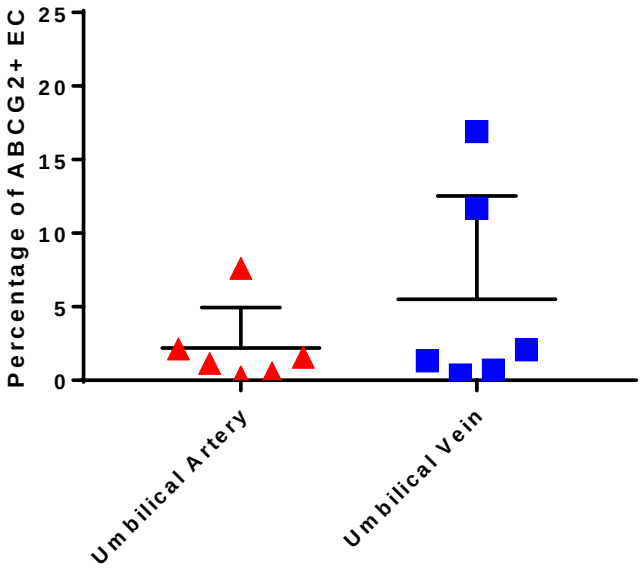
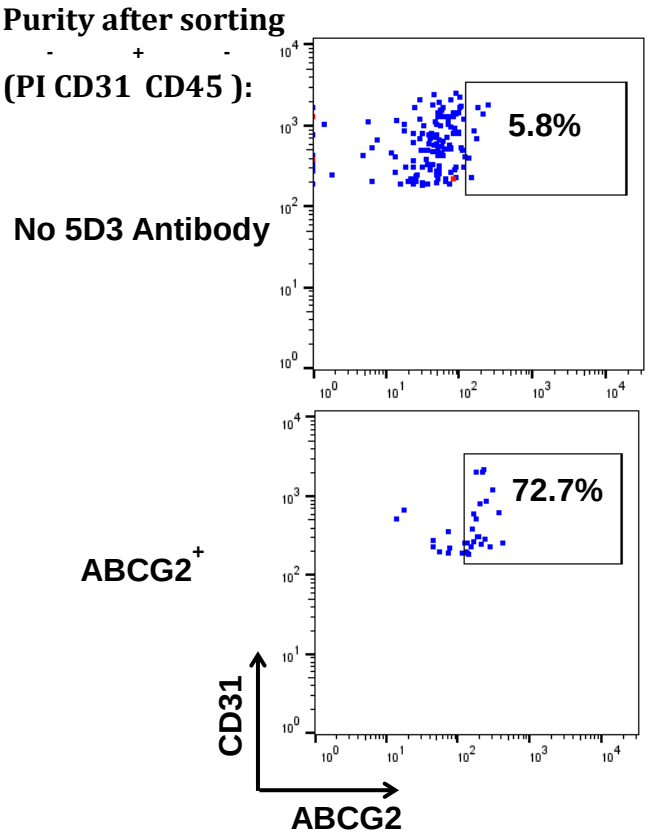
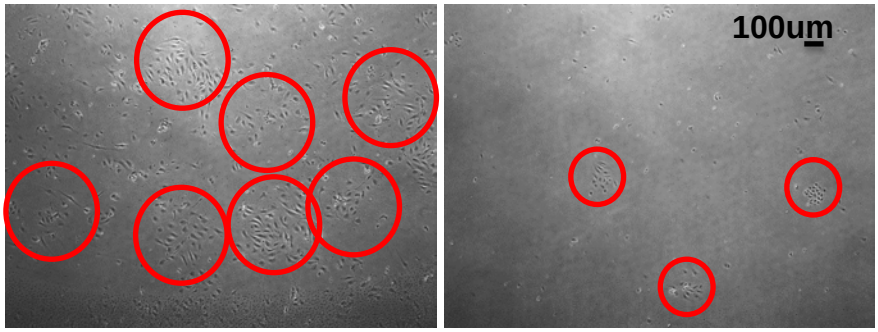


Figure II. 32. Human blood vessel contain ABCG2 expressing EC. (A). Representative pictures show the distribution of ABCG2$^+$ EC in human umbilical artery (top panels) and vein (bottom panels). Red, ABCG2; Green, CD31; Blue, DAPI. (B). Flow cytometry data show the percentage of ABCG2 $^+$ EC in primary human umbilical artery (HUAEC) or vein (HUVEC) EC. (C). Quantitation of data from B.

A


Purity after sorting
(PI- CD31+ CD45-):
No 5D3 Antibody
5.8%
ABCG2+
72.7%
CD31
ABCG2

B


100um

C

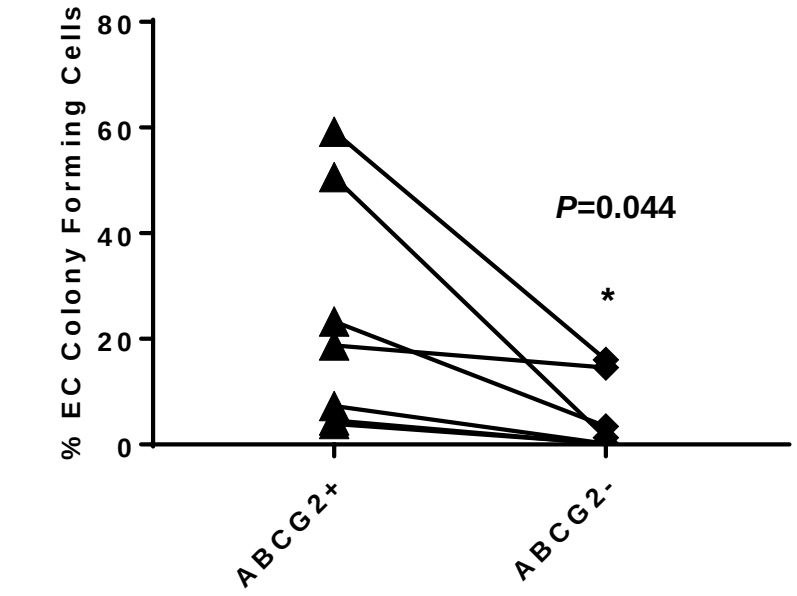
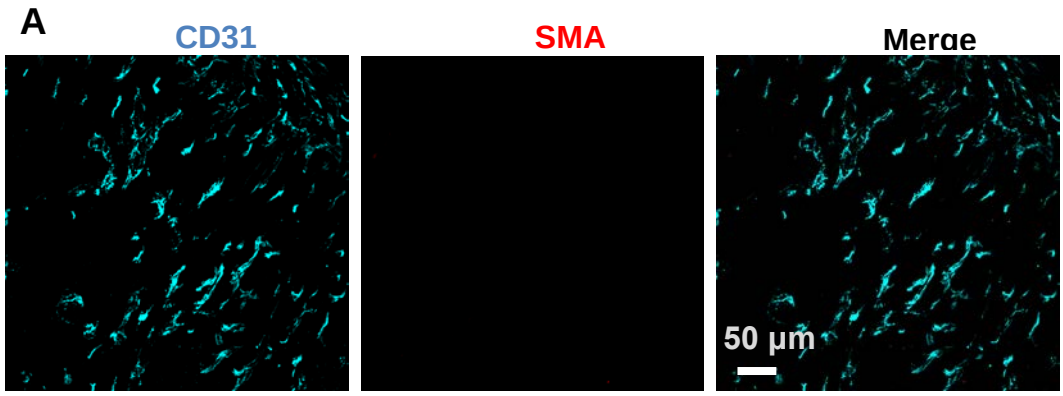
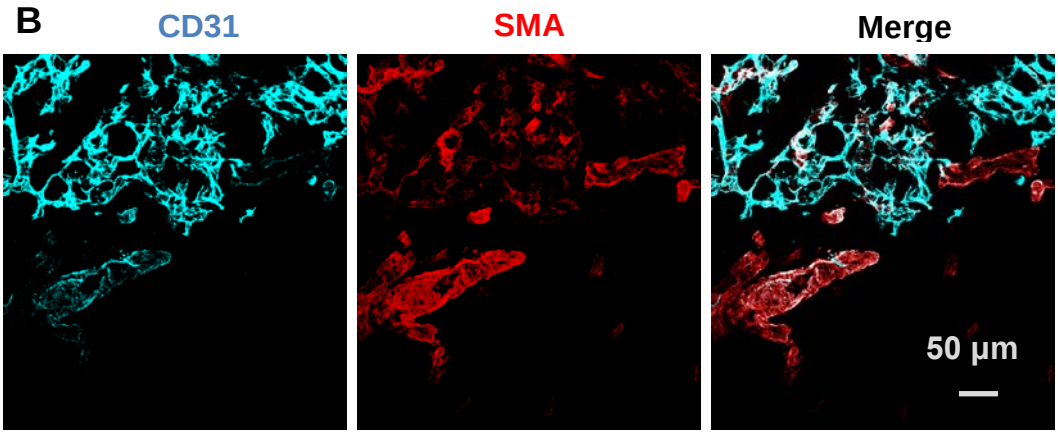


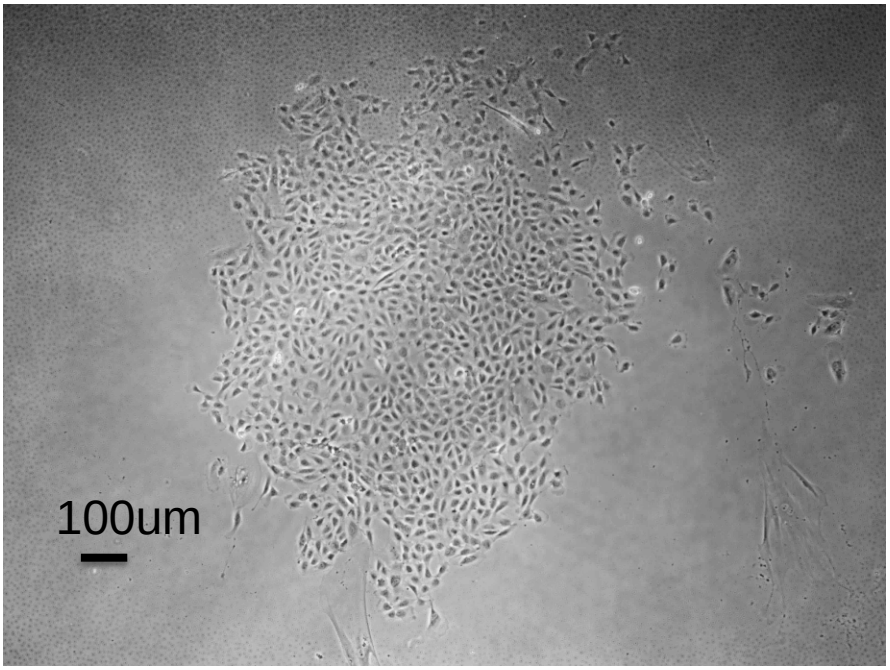
Figure II. 33. Human ABCG2 expressing VESC have *in vitro* colony forming potential. (A). Purity of magnetic activated cell sorting separated ABCG2^{+} HUVEC tested by flow cytometry. (B). Representative pictures of EC colonies derived from ABCG2^{+} HUVEC (150 EC on 1 well of 6 well plate, left panel) and ABCG2^{-} HUVEC (1, 000 EC on 1 well of 6 well plate, right panel). (C). Percentage of EC colony forming cells in freshly isolated ABCG2^{+} and ABCG2^{-} CD31^{+}CD45^{-} HUVEC. Data represent mean ± s. d. *p* values, two-tailed paired t-test. (*n*=7 patients from 5 independent experiments).

We isolated single ABCG2$^+$ HUVEC derived cells, expanded them *in vitro*, and transplanted each clone of ABCG2$^+$ EC derived cells into NOD/SCID mice in the presence of OP9 stromal cells (4:1 ratio of EC to OP9) or OP9 cells that express NOTCH ligand DL-1 (OP9-DL1, 4:1 ratio of EC to OP9-DL1) which we have previously reported to promote transplanted EC to adapt an arterial EC phenotype (Kim et al., 2015). After 2 weeks of implantation, robust blood vessels were identified in all recovered gels (*n*=4, Figure II. 34A, B). While vessels from gels in which ABCG2+ HUVEC-derived EC were co-transplanted with OP9 displayed a capillary morphology (Figure II. 34A), single ABCG2$^+$ HUVEC derived EC formed both capillaries and arteries in OP9-DL1 co-transplanted gels (Figure II. 34B). Additionally, when single ABCG2$^+$ HUVEC derived EC implanted gels were digested and re-plated, cobble-stone like secondary EC colonies were discovered from the culture (Figure II. 34C), and these cells could be re-implanted into secondary recipient mice to generate donor vasculature (Figure II. 34D), demonstrating that ABCG2$^+$ VESC, at a clonal level have the potential to self-renew. As further confirmation, In sum, like murine *Abcg2*-expressing VESC, human ABCG2$^+$ EC have the potential for clonal expansion *in vitro*, give rise to EC comprising capillaries and macrovessels *in vivo*, self-renew *in vivo* to form primary and secondary blood vessels, and thus fulfill the criteria of resident VESC.


A
CD31
SMA
Merge
50 µm


B
CD31
SMA
Merge
50 µm

C



hCD31

D

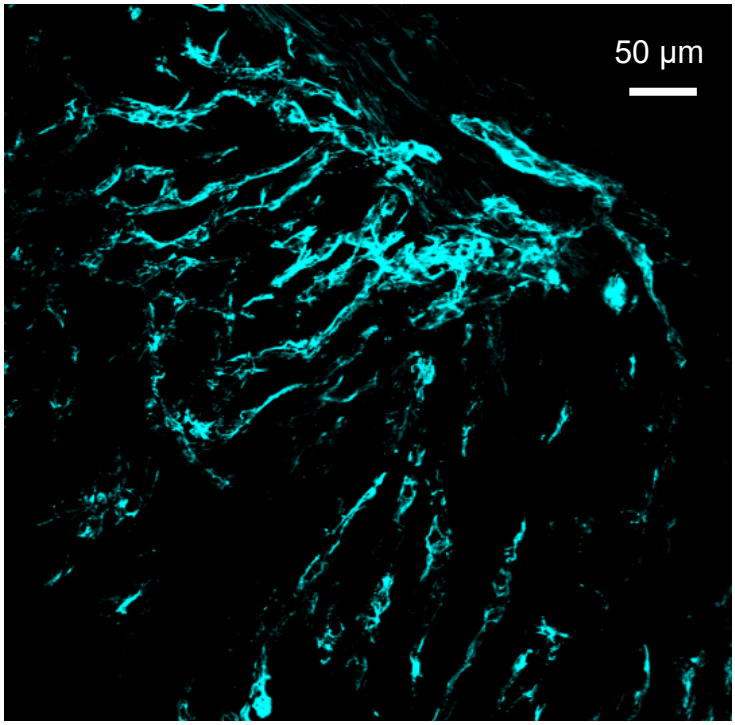


Figure II. 34. Human ABCG2 expressing VESC have *in vivo* vessel forming potential. (A). Single ABCG2$^+$ HUVEC derived arteries and capillaries 2 weeks after co-transplantion with OP9. Cyan, human CD31; Red, smooth muscle actin α (SMA). (B). Single ABCG2$^+$ HUVEC derived arteries and capillaries 2 weeks after co-transplantation with OP9-DL1. Cyan, human CD31; Red, smooth muscle actin α (SMA). (C). Representative pictures of a secondary HUVEC colony derived from single ABCG2$^+$ HUVEC transplanted gel. (D). Representative picture of secondary blood vessels in host mouse derived from secondary colonial EC retrieved from single ABCG2$^+$ HUVEC transplanted gel. Cyan, human CD31.

4. Discussion

Resident stem cells are been known to be present in numerous healthy and malignant tissues and support the growth and maintenance of cells from various lineages including hematopoietic cells (Eaves, 2015; Lin et al., 2014), intestine epithelial cells (Barker, 2014), neural cells (Bhattacharya et al., 2007; Conti and Cattaneo, 2010) and cancer cells (Nassar and Blanpain, 2016). While several groups have identified some rare immature EC that possess greater proliferative potential than mature EC (Alphonse et al., 2015; Canete et al., 2017; Fang et al., 2012; Ingram et al., 2005; Ingram et al., 2004; Javed et al., 2008; Kusumbe et al., 2014; Malinverno et al., 2017; Naito et al., 2012; Naito et al., 2016; Shelley et al., 2012; Yu et al., 2016), and a number of cardiovascular diseases have been linked with abnormal resident and circulating EC colony forming ability (Alvarado-Moreno et al., 2016; Lee et al., 2015; Prasain et al., 2014), the concept of vascular endothelial stem / progenitor cells has not been widely appreciated. No reports have identified a marker that permits prospective identification of VESC in mouse and man. Here we have identified that the expression of ATP binding cassette transporter member Abcg2 (ABCG2 in human) labels VESC in both mouse and man, and proved that these cells fulfill all criteria of true stem cells that have been adapted from the definition of other lineage-specific stem cells, like hematopoietic stem cells (Eaves, 2015). Indeed, *ABCG2*-expressing VESC possess clonal *in vitro* EC colony forming potential and the ability to differentiate to artery, vein and capillary EC *in vivo.* In addition, both human and murine VESC can self-renew by maintaining their colony forming potential as well as vessel forming potential upon primary and secondary transplantation. Thus, in our lineage tracing model

using *Abcg2*CreERT mice, rare *Abcg2*-expressing VESC in P1 mice were found to significantly contribute to vessel growth/maintenance in multiple organs include heart, lung, bone, retina, and skeletal muscle for up to 10 months. Interestingly, in adult mice, *Abcg2*-expressing VESC persist, though at a limited frequency compared to the immediate postnatal period and have the potential to participate in vessel regeneration after injury.

Abcg2 (ABCG2) has been identified to be highly expressed in many lineage-specific stem cells (Bhattacharya et al., 2007; Fatima et al., 2012; Moitra, 2015; Tadjali et al., 2006; Zhou et al., 2001) and pluripotent stem cells (Apati et al., 2008). In addition, although ABCG2 is not likely to be a driver of stemness, since overexpression of ABCG2 does not improve, but in fact disrupts some stem cell functions (Bhattacharya et al., 2007; Ueda et al., 2005; Wee et al., 2016; Zhou et al., 2001), it is crucial for the maintenance of various stem cells, including VESC, from xenobiotic and cytotoxicity stress (Apati et al., 2008; Bhattacharya et al., 2007; Chen et al., 2010; Zhou et al., 2002; Zhou et al., 2003). In the EC lineage, it is known that knock-out of *Abcg2* leads to compromised vessel growth / regeneration upon injuries(Doyle et al., 2011; Higashikuni et al., 2010; Higashikuni et al., 2012; Maher et al., 2014), while the mechanism for this phenomenon has not been previously elucidated. In this study, we have discovered that *Abcg2*-expressing VESC, which represent a large fraction of EC colony forming cells in multiple neonatal and adult tissues, contribute substantially to vessel growth during development. In addition, we have found that the function of Abcg2 is crucial for the maintenance of colony EC forming potential.

Thus, for those cardiovascular disease patients with diminished vascular EC proliferative potential, like peripheral artery disease (PAD) patients(Prasain et al., 2014), ABCG2 is a potential target to identify resident VESC to better define the pathophysiologic mechanisms in PAD and develop potential strategies for the use of ABCG2 expressing VESC for PAD treatment.

Chapter III

Circulating Endothelial Stem Cells are Derived from Vascular Endothelial Cells and Can Participate in Blood Vessel Formation *in vivo*

1. Introduction

While the angiogenic growth of new vessels from pre-existing vessels is well defined, little is known of the cellular and molecular determinants of EC replacement and repair within a blood vessel. An improved understanding of EC replacement and repair is required to develop new methods to promote the recruitment or capture of resident or circulating endothelial cells (CEC) to implanted vascular stents or small diameter tissue engineered vascular segments to prevent vessel restenosis or clotting, respectively. The ability of viable CEC to adhere to and proliferate to cover exposed intravascular surfaces of implanted materials or devices has been known for more than 50 years (Stump et al., 1963), however, the origin of the viable CEC remains controversial.

The vast majority of human CEC are quiescent, apoptotic, or necrotic cells that are increasingly being used as a quantitative biomarker to assess the presence of vascular inflammation, injury, or dysfunction in numerous human diseases. Rare viable CEC that grow from cultured peripheral blood cells have also been widely reported in healthy human subjects and those with disease (Ingram et al., 2004; Javed et al., 2008). The ability of some individually cultured viable CEC to produce colonies of EC progeny has led to their designation as circulating endothelial colony forming cells (cECFC). Human cECFC display a hierarchy of clonal proliferative potential (from

clusters of 2-50 cells to colonies of >2000 EC), undergo massive expansion in vitro (up to 10^{13} progeny per clone), form human blood vessels upon implantation in immunodeficient mice, and play robust roles as a cell therapy in the repair or regeneration of blood vessels in multiple animal models of human disease(Ingram et al., 2004; Javed et al., 2008). The origin of viable human CEC has been attributed to the release from the endothelial intima in blood vessel walls (Ingram et al., 2005; Yoder, 2010) but, evidence that they arise from engrafting donor bone marrow precursors following a bone marrow transplant has also been reported (Lin et al., 2000). Thus, the precise origin of human cECFC remains controversial.

Here we tested two hypotheses: 1. Murine cECFC are derived from vascular EC but not hematopoietic cells; 2. Murine cECFC represent circulating endothelial stem cells (CESC). Our lineage tracing data show that murine cECFC are progeny of vascular EC, but are not derived from hematopoietic lineage. Additionally, cECFC have the potential to form EC colonies *in vitro*, form functional blood vessels upon transplantation, and possess the ability to self-renew *in vivo*. Thus cECFC fulfill the criteria of CESC. Finally, we showed that like resident VESC, CESC also express *Abcg2*, and the production of CESC can be diminished by the loss of *Abcg2*.

2. Materials and methods

Animals

All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, and all protocols were approved by Institutional Animal Care and Use Committee of the Indiana University School of Medicine. C57BL/6 (JAX stock #000664), 129S1/SvImJ (SV129, JAX stock #002448), FVB/NJ (FVB, JAX stock #001800), B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J (Madisen et al., 2010) (R26R-TdTomato, JAX stock #007914), Gt(ROSA)$^{26Sortm1.1(CAG-EGFP)Fsh}$/Mmjax (Sousa et al., 2009) (R26R-EGFP, MMRRC stock #32037), NOD.CB17-Prkdc^{scid}/J (NOD/SCID, JAX stock #001303) were purchased from the Jackson Laboratory. Bcrp constitutive knock out mice (ABCG2 knockout, #2767) were obtained from Taconic. CD1 mice (#022) were purchased from Charles River Laboratories. Flt3Cre^{+};ROSA$^{mTmG/mTmG}$ mice (Benz et al., 2008; Epelman et al., 2014; Muzumdar et al., 2007) were a kind gift from Dr. Slava Epelman, University of Toronto. Cdh5(PAC)-CreERT2 mice (Wang et al., 2010) were a kind gift from Dr. Ralf Adams, Max Planck Institute for Molecular Biomedicine, Germany. The generation of Tie2CreER^{T2} mice are described in the next section.

Generation of Tie2CreER^{T2} mice

The DNA fragment containing a β-globulin gene intron sequence, Cre recombinase, mutated estrogen receptor ligand binding domain and SV40 polyA (klenow enzyme filled Stu I site – Xba fragment) was excised from plasmid pCreER^{T2} (Feil et al., 1997) and was inserted into the EcoRV-Xba I site in plasmid pBSTie2-2 (You et al., 2005) to generate plasmid pTie2P-CreER^{T2}. Next, the Sal1 DNA fragment, which contains the

Tie2 promoter region and CreERT2, was excised from pTie2P-CreER^{T2} plasmid and inserted into pBSpolyATie2-10 (You et al., 2005) upstream of SV40 polyA signal and the first intron sequence. Finally, the Tie2-CreER^{T2} transgene, which contains Tie2 promoter, β-globulin intron, CreER^{T2}, 2 polyadenylation signals and Tie2 enhancer was excised from the vector and used for pronuclear microinjection into C57BL/6 to generate Tie2CreER^{T2} mice.

To induce cre expression in Tie2CreER^{T2}, Cdh5(PAC)-CreERT2, and ABCG2CreERT mice, 50mg/kg tamoxifen was injected into the animals intra-peritoneally (i.p.) at appropriate time points. To block Abcb1a and Abcb1b transporter, 50mg/kg tetrandrine was i.p. injected daily into pups for 4 consecutive days.

Cell collection

Blood was collected from postnatal mice by cardiac puncture. After blood collection, mononuclear cells were isolated by re-suspending in red blood cell lysis buffer (Qiagen) for 10 minutes (for cECFC culture) or density gradient centrifugation using Histopaque 1083 (for flow cytometry).

To collect cells from lung and heart, tissues were minced and then digested with 0.25% collagenase I (Stem Cell Technologies) at 37°C for 30 minutes. The digested samples were passed through 70μm cell strainers to removed cell clumps. Bone marrow cells were collected by flushing the tibia or femur bones with PBS using a syringe and 21G needle.

Flow cytometry

The following anti-mouse antibodies conjugated with different fluorochrome were used for flow cytometry sorting and analysis: CD31 (clone 390), CD144 (11D4.1), Tie2 (TIE2), CD45 (30-F11), Ter119 (TER119), PSGL1 (FLEG), B220 (RA3-6B2), CD3 (145-2C11), CD11b (M1/70), CD150 (9D1), CD48 (HM48-1), Scal1 (D7), c-Kit (2B8) (all above antibodies were purchased from eBioscience). For hematopoietic lineage staining, the cells were stained with BD Biosciences Biotin Mouse Lineage Panel (CD3e, B220, Ly6G/Ly-6C, CD11b, Ter119) first and then stained with streptavidin conjugated secondary antibodies (clone, BD Biosciences). 1:1000 propidium iodide (PI, Sigma-Aldrich) was added to sorting buffer before analysis to distinguish live and dead cells. Cell analysis and sorting were performed on LSR 4, LSRII, FACSCantoII, FACSAria, and SORPAria flow cytometers (BD Biosciences). FlowJo software was used to analyze flow cytometry data. For single cell culture, cells were sorted and each cell was directly loaded into each well of 96 well plated coated with OP9 stromal cell monolayer. For endothelial cell colony forming limiting dilution assay, 20, 50, 100 P6 heart TdTomato+ endothelial cells, or 200, 500, and 1000 P6 lung TdTomato+ endothelial cells were sorted into 1 well of OP9-coated 96 well plates, respectively.

Culture of Endothelial Colonies

OP9 stromal cells were maintained in OP9 medium (alpha-MEM medium [Gibco], with 20% FBS, and 0.5% penicillin/streptomycin [Gibco]). To culture endothelial colonies, isolated endothelial cells or peripheral blood mononuclear cells were re-

suspended in EC culture medium (alpha-MEM with 10% FBS, 5×10^{-5} M β-mercaptoethanol and 0.5% penicillin/streptomycin). After 24 hours, non-adherent cells were removed by changing with fresh medium. Medium was changed every 3 days afterwards until use.

In vivo Gel implantation

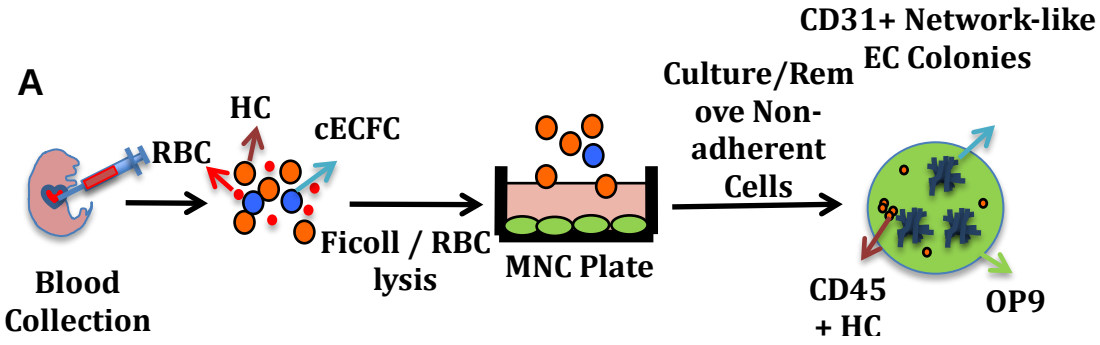
Blood from tamoxifen injected Tie2cre; ROSATdTomato pups were culture on OP9 for 3-8 weeks. Then the cultured EC were collected and minced with a razor blade before mounting into collagen gels. Culture cells from 1 pup were re-suspended in 250ul 200pa collagen gel (Geniphys, Standarized Oligomer Polymerization Kit) plus 10% human platelet lysate (Cook) on ice and transferred into 1 well of 48 well plate. The gel was placed in a 37 ˚C incubator to polymerize for 30 minutes. Next the cellularized gels were transplanted subcutaneously into the flanks of 6-12 weeks old NOD/SCID mice. The gels were retrieved from the animals at various time points between 14 days and 10 months post-implantation. To test the vessel forming potential of un-cultured mouse or human circulating ECFC, blood from 10 P3 Tie2Cre; ROSATdTomato pups (tamoxifen injected on P0, P1, P2), or MACS isolated 300, 000 human cord blood $CD34^+$ cells were each suspended into a single collagen gel.

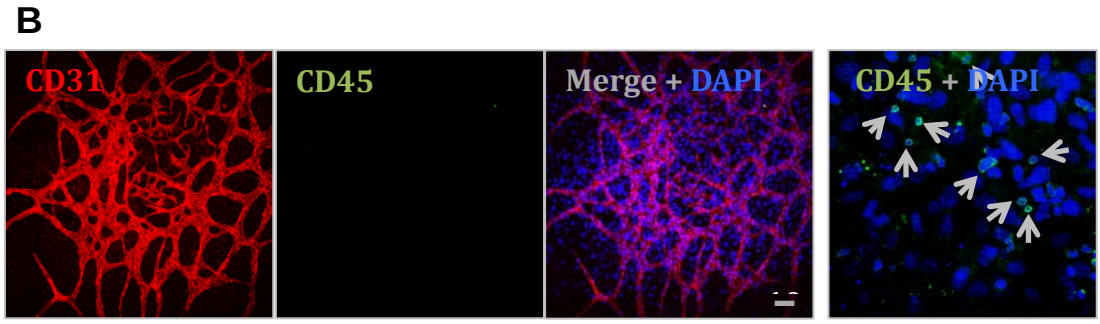
All protocols used for imaging and statistical analysis are same with the methods described in Chapter II (page 38-42).

3. Results

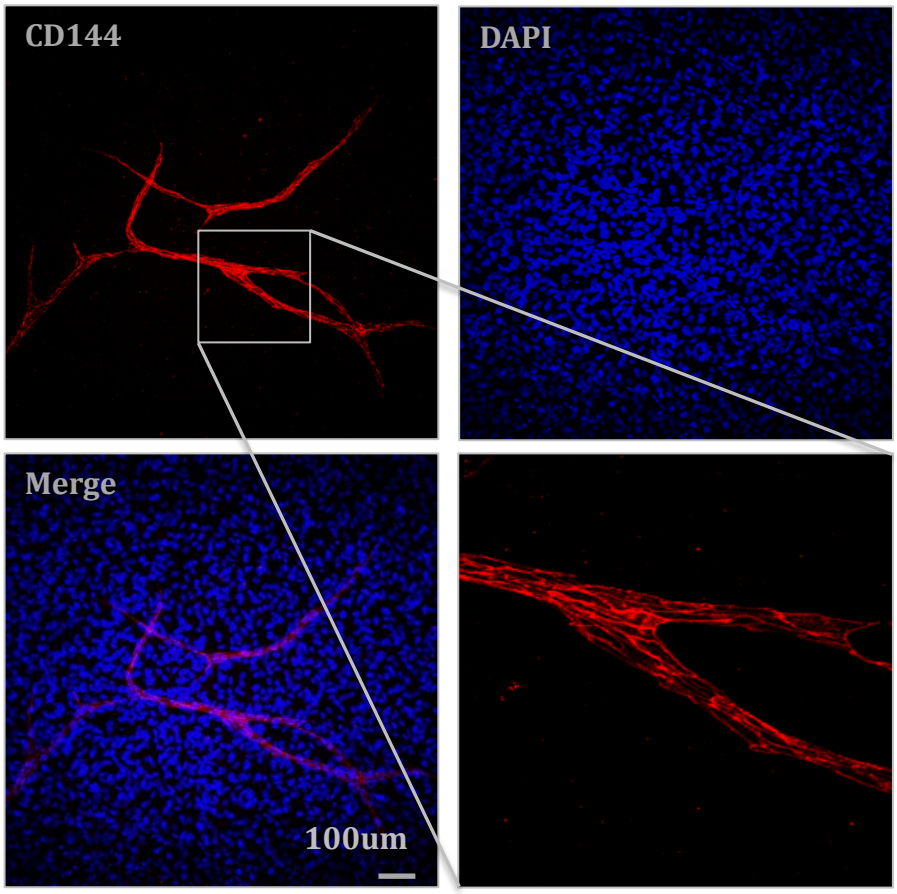
Neonate/juvenile murine peripheral blood contains cECFC

While cECFC are essentially undetectable in individual adult mice (Somani et al., 2007), we hypothesized that cECFC may be present in the bloodstream of newborn mice, given that human cECFC are enriched 60 fold in newborn umbilical cord blood (Ingram et al., 2004; Javed et al., 2008). To enhance our ability to identify murine cECFC, we employed a method of co-culturing the peripheral blood low density mononuclear cells (MNC) over a monolayer of OP9 murine bone marrow stromal cells; a method previously successful in growing EC colonies from differentiated murine embryonic stem cells or selected adult organ vasculature (Naito et al., 2012; Naito et al., 2016). Within 4 days of culture, compact EC colonies in "sheets" or network-like morphology appeared (Figure III. 1); similar in appearance to previously reported EC colonies grown on OP9 (Hashimoto et al., 2007; Hirashima et al., 1999; Naito et al., 2012; Naito et al., 2016; Wakabayashi et al., 2013). These colonies expressed the known EC surface markers CD31, CD144 and Flk1 (Figure III. 1B-D, Figure III. 2), exhibited typical endothelial *in vitro* properties of acetylated low density lipoprotein (AcLDL) binding and lectin uptake (Figure III. 1E, F), but did not express hematopoietic cell surface markers CD45 and CD11b (Figure III. 1B, C).

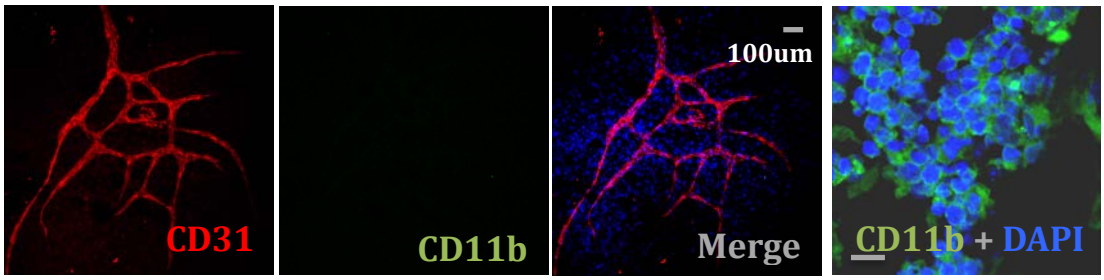

A
RBC
HC
cECFC
Blood Collection
Ficoll / RBC lysis
MNC Plate
Culture/Remove Non-adherent Cells
CD31+ Network-like EC Colonies
CD45 + HC
OP9


B
CD31
CD45
Merge + DAPI
CD45 + DAPI

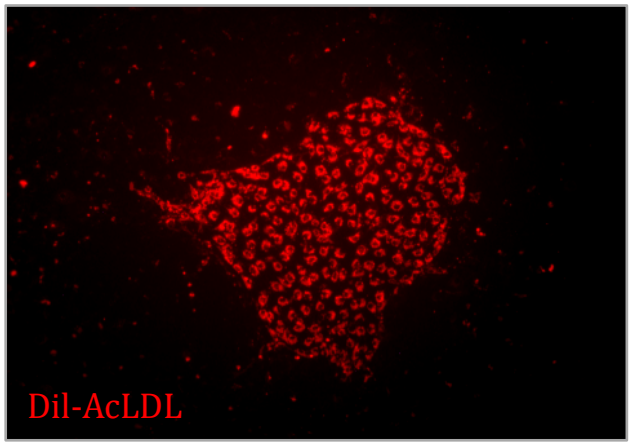
C



D



E



F

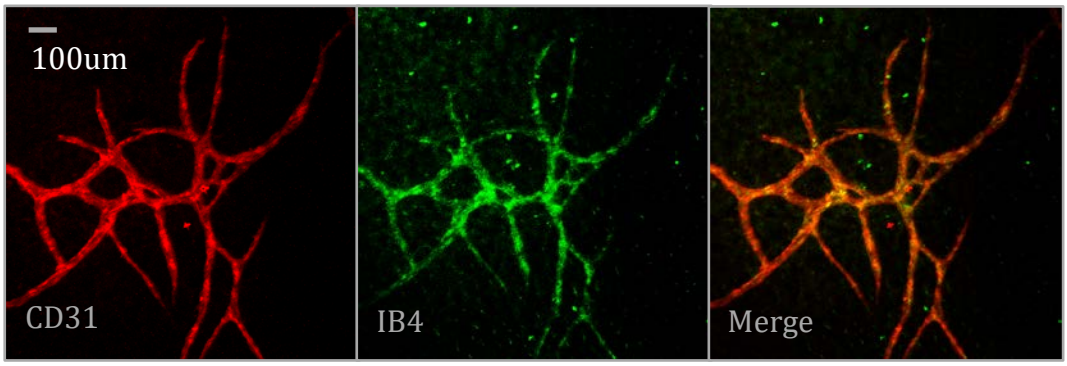


Figure III. 1. Murine blood contain cECFC. (A). Schematic of cultured cECFC from murine peripheral blood. (B). Murine cECFC derived EC colonies express the endothelial marker CD31 (red), but not the hematopoietic marker CD45 (green). (C). Murine cECFC derived EC colonies express the endothelial marker CD144. (D). Murine cECFC derived EC colonies do not express the myeloid hematopoietic marker CD11b (green). (E). Murine cECFC derived EC colonies can ingest AcLDL. (F). Murine cECFC derived EC colonies can bind lectin (green). IB4, isolectin B4. HC, hematopoietic cells; RBC, red blood cells

Isolation of cECFC from newborn murine blood was confirmed in C57/BL6, FVB, CD1 and SV129 mice (Figure III. 2). The cECFC number peaked right after birth and declined rapidly afterwards (Figure III. 3). By 22 days of age, cECFC were undetectable from C57/BL6 murine blood, but remained present at a low level in FVB murine blood for 3 months (Figure III. 3), suggesting the kinetics of cECFC emergence in each strain has some variation, but loss of cECFC from the blood of adult mice is a common trait.

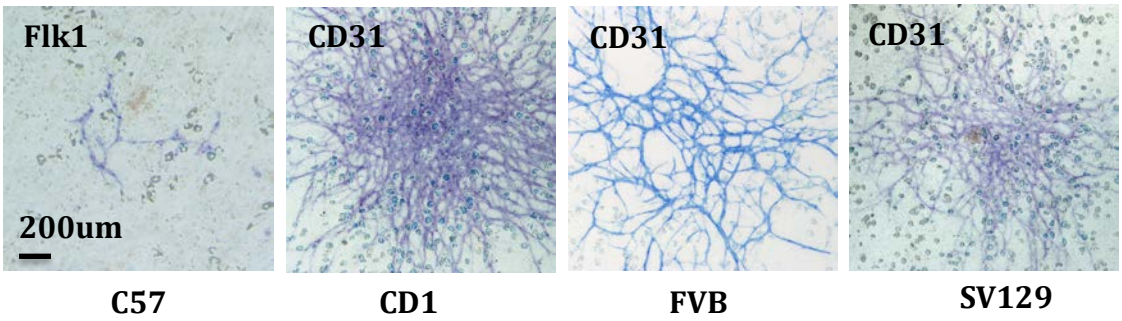


Figure III. 2. cECFC derived colonies from the peripheral blood of C57, CD1, FVB and SV129. Pictures show the staining of alkaline phosphatase conjugated anti-rat IgG secondary antibodies against rat anti mouse Flk1 (left panel), or CD31 (right 3 panels).

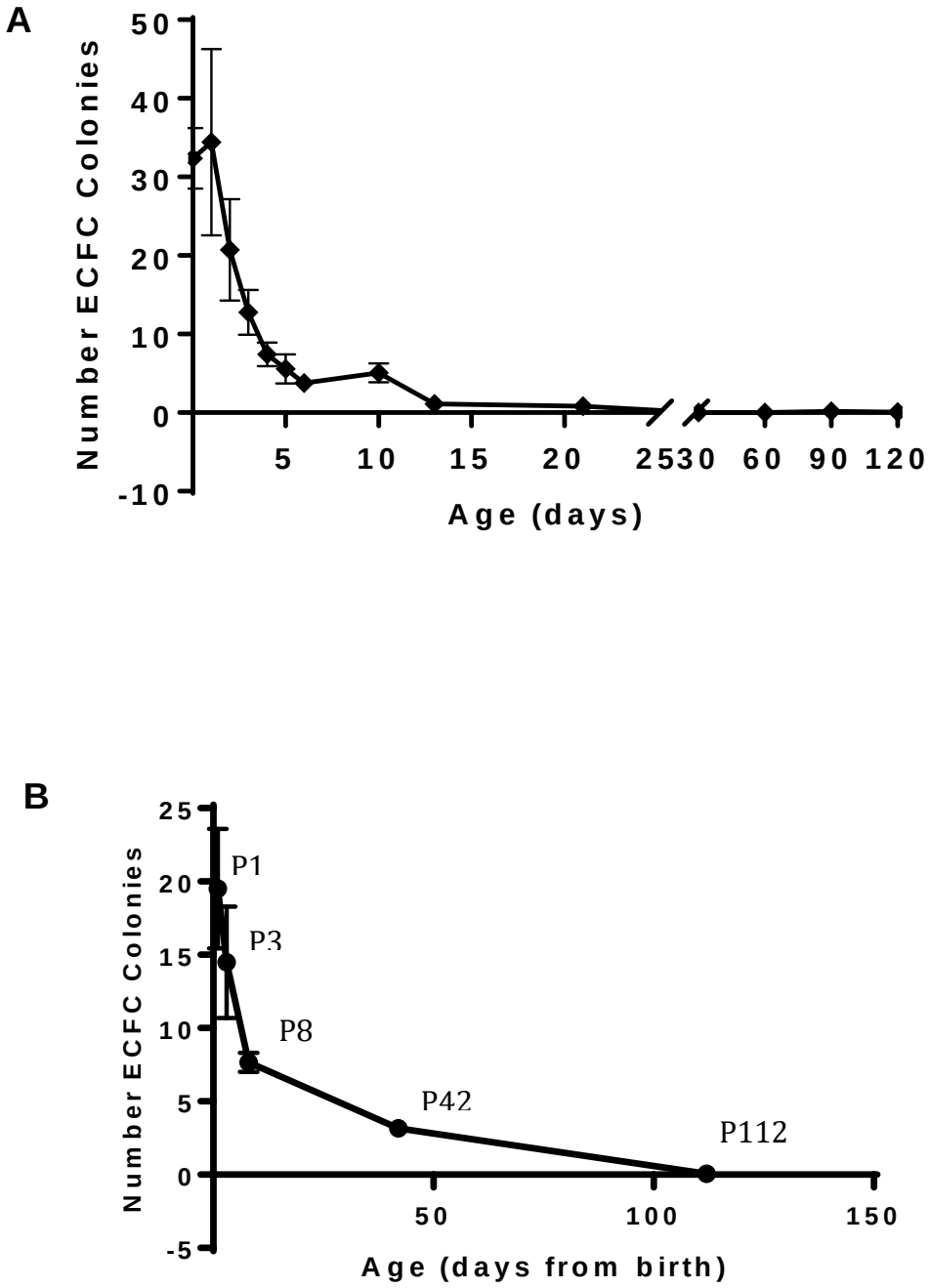
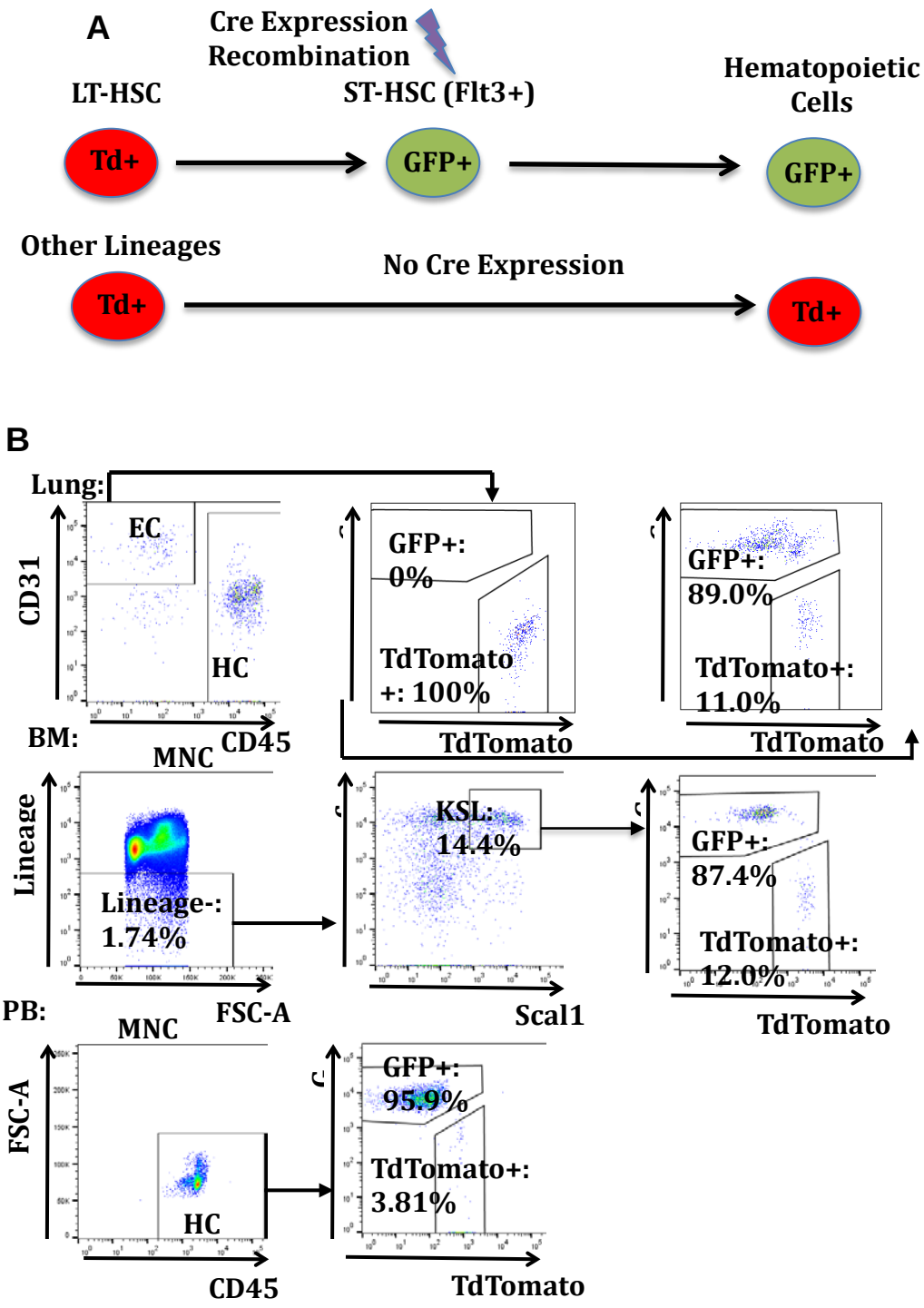


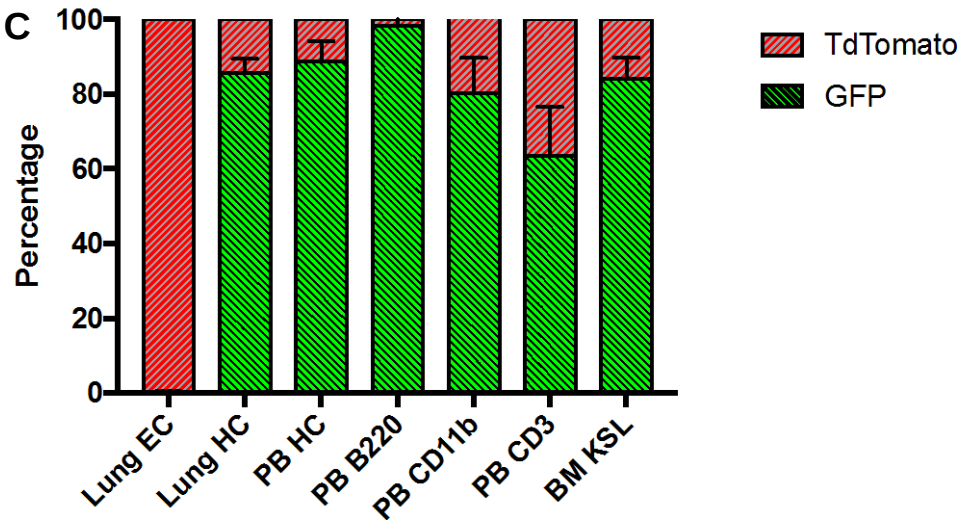
Figure III. 3. Kinetics of emergence of cECFC in C57 (A) and FVB (B) mice blood.

Murine cECFC are derived from vascular EC but not hematopoietic cells

cECFC are derived from vascular endothelial cells

To assess whether the cECFC present in the newborn mouse blood arose from a bone marrow hematopoietic precursor or from resident vascular EC, we performed lineage tracing using Flt3cre; mTmG (Flt3TG) mice, in which all hematopoietic stem cell (HSC) derived progeny are labeled with green fluorescence protein (GFP) (Boyer et al., 2011)(Figure III. 4A) and confirmed that 85-90% of hematopoietic cells and bone marrow c-kit$^+$Scal1$^+$lineage$^-$ (KSL) progenitor cells were GFP+ (Figure III. 4B, C). All lung EC failed to express GFP (Figure III. 4B, C), confirming that resident EC were not derived from hematopoietic cells during development. Next, we plated peripheral blood MNC from Flt3TG mice on OP9 cells and found all 99 cECFC colonies derived from 12 mice were TdTomato+, and none expressed GFP (Figure III. 4D), proving that murine cECFC are not derived from bone marrow hematopoietic cells.


A
Cre Expression
Recombination
LT-HSC
ST-HSC (Flt3+)
Hematopoietic Cells
Td+
GFP+
GFP+
Other Lineages
No Cre Expression
Td+
Td+
B
Lung:
CD31
EC
HC
CD45
GFP+: 0%
TdTomato +: 100%
TdTomato
GFP+: 89.0%
TdTomato+: 11.0%
TdTomato
BM:
MNC
Lineage
Lineage-: 1.74%
FSC-A
KSL: 14.4%
Scal1
GFP+: 87.4%
TdTomato+: 12.0%
TdTomato
PB:
MNC
FSC-A
HC
CD45
GFP+: 95.9%
TdTomato+: 3.81%
TdTomato


C
100
80
60
40
20
0
Percentage
TdTomato
GFP
Lung EC
Lung HC
PB HC
PB B220
PB CD11b
PB CD3
BM KSL

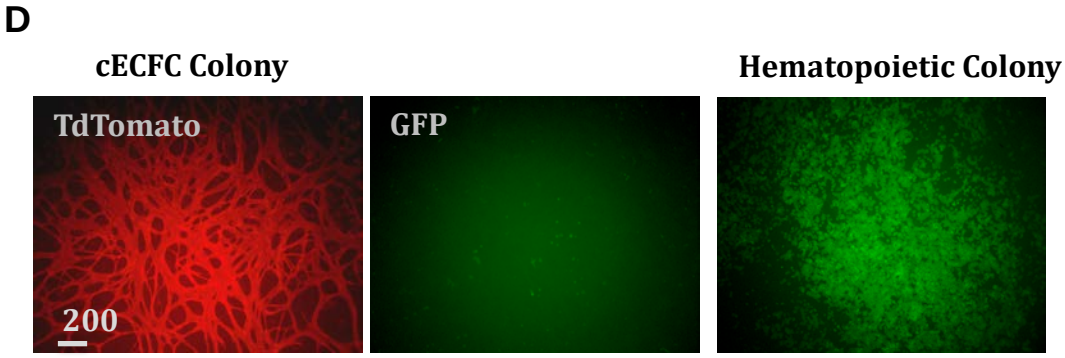
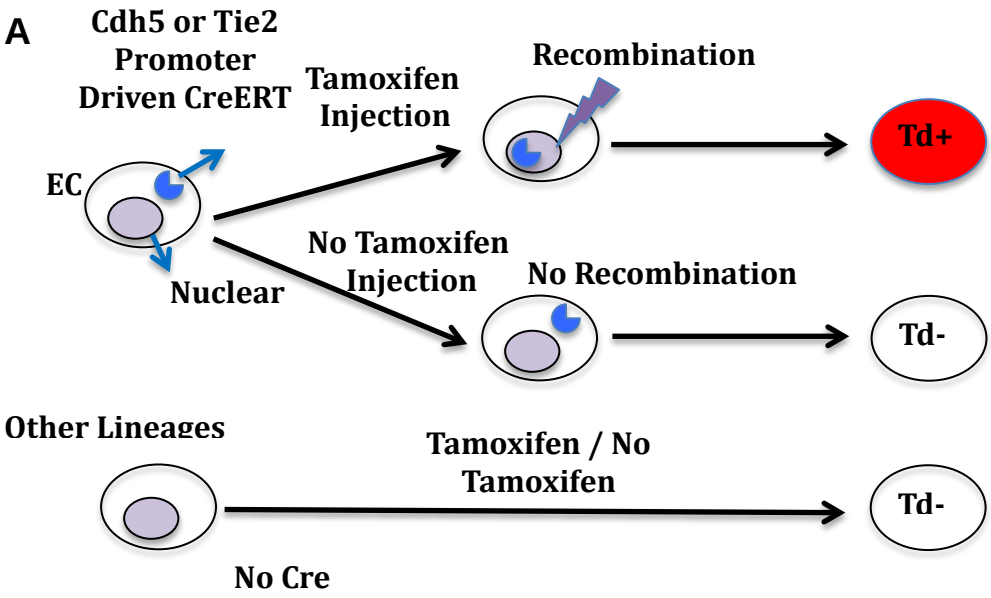
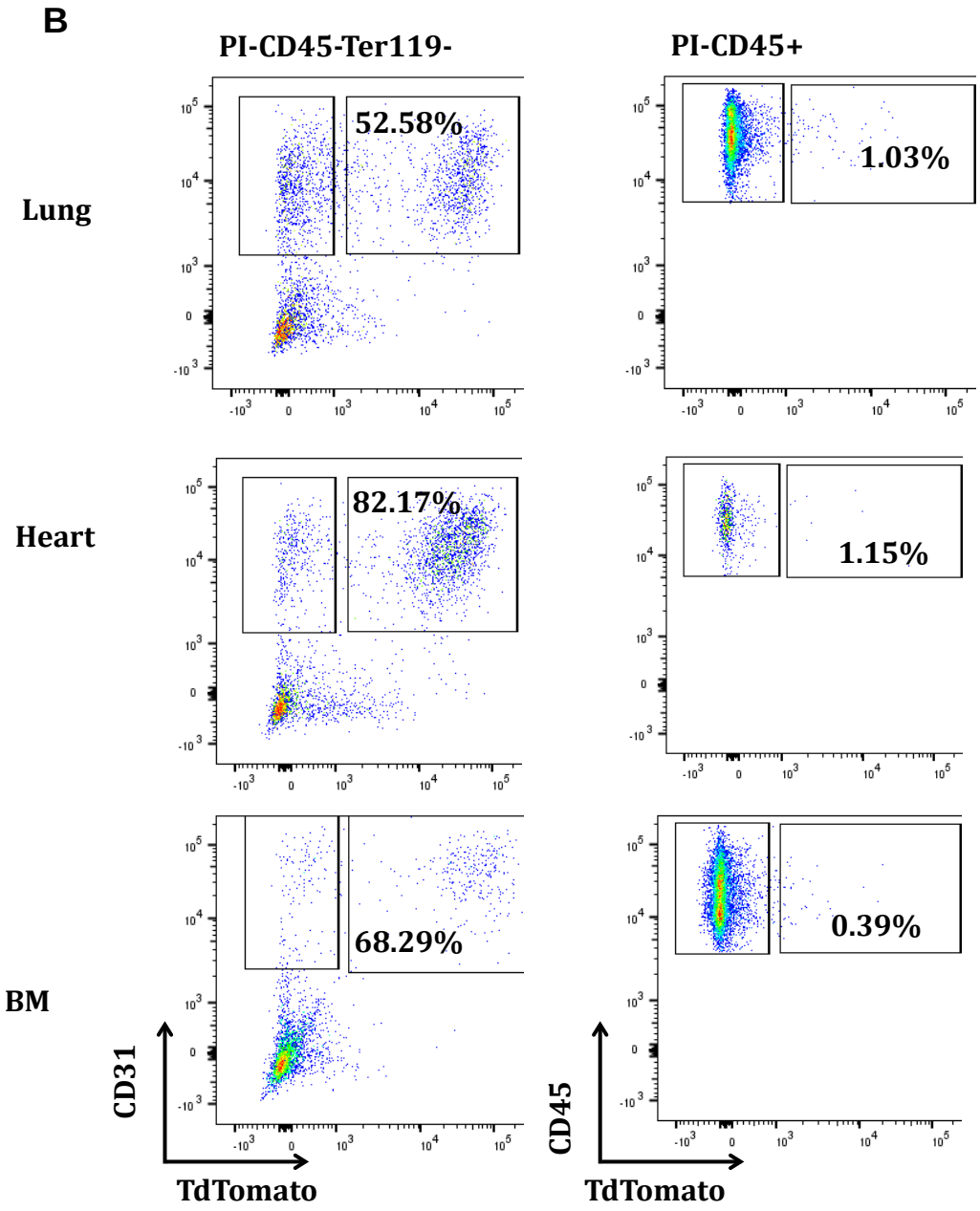

D
cECFC Colony
Hematopoietic Colony
TdTomato
GFP
200

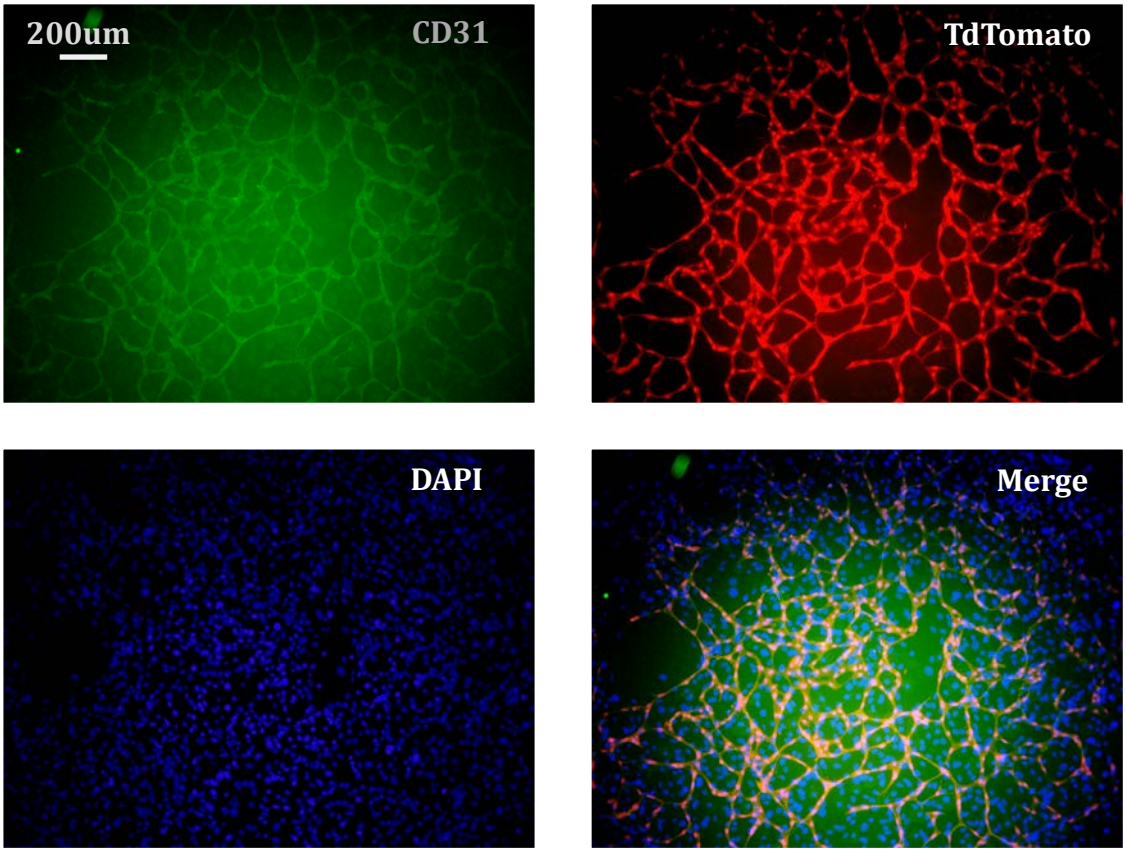
Figure III. 4. cECFC are not derived from hematopoietic cells. (A). Schematics of lineage tracing using Flt3Cre;mTmG mice. (B). Flow cytometry analysis of postnatal day 14 Flt3Cre;mTmG mice. GFP did not label EC in the lung but labeled almost all hematopoietic cells and hematopoietic stem cells in the lung, bone marrow and peripheral blood. (C). Quantitation of data from (C). (D). cECFC (left two panels) and hematopoietic (right panel) colonies derived from peripheral blood of Flt3Cre;mTmG mice. 99 circulating ECFC Colonies from 25 pups were all negative for GFP.

To test if cECFC are derived from resident vascular EC, we crossed Cdh5(PAC)-CreERT2 (Wang et al., 2010), an endothelial specific driver, with Rosa26 floxed-stop TdTomato (ROSATT) reporter mice to generate Cdh5TT mice. Administration of tamoxifen at postnatal day (P) P0-P2, labeled the majority of tissue resident EC from P3 mice (Figure III. 5A, B). In co-culture with OP9, 52 of 56 (93%) PB cECFC colonies from P3 Cdh5TT mice expressed TdTomato (Figure III. 5C), suggesting cECFC are derived from resident EC. As a confirmation, we generated novel Tie2CreERT mice in which Cre expression is restricted to EC (Figure III. 6). When Tie2CreERT mice were crossed with R26REGFP or ROSATT mice and received tamoxifen injections on P0-P3, >50% of EC in different tissues were specifically labeled on P4 (Figure III. 6). PB MNC isolated from Tie2TT mice after postnatal tamoxifen labeling gave rise to 155 out of 177 (88%) cECFC colonies to TdTomato$^+$ cells (Figure III. 6B, C), confirming that murine cECFC are derived from resident EC. To exclude the possibility that cECFC were labeled while circulating in the blood stream, we collected blood MNC and tissue resident EC from un-activated Tie2CreTT mice and incubated them in 4 hydroxyl-tamoxifen (4-OHT) for 8 hours before plating in OP9 co-culture (Figure III. 6D). While nearly half of the activated lung and heart resident EC expressed TdTomato, cECFC colonies were not labeled during the *in vitro* exposure to 4-OHT (Figure III. 6E, F). Thus, cECFC must arise from resident EC prior to release into the blood stream.


A
Cdh5 or Tie2
Promoter
Driven CreERT
Tamoxifen
Injection
Recombination
Td+
EC
Nuclear
No Tamoxifen
Injection
No Recombination
Td-
Other Lineages
Tamoxifen / No
Tamoxifen
Td-
No Cre


B
PI-CD45-Ter119-
PI-CD45+
Lung
Heart
BM
52.58%
1.03%
82.17%
1.15%
68.29%
0.39%
CD31
TdTomato
CD45
TdTomato

C



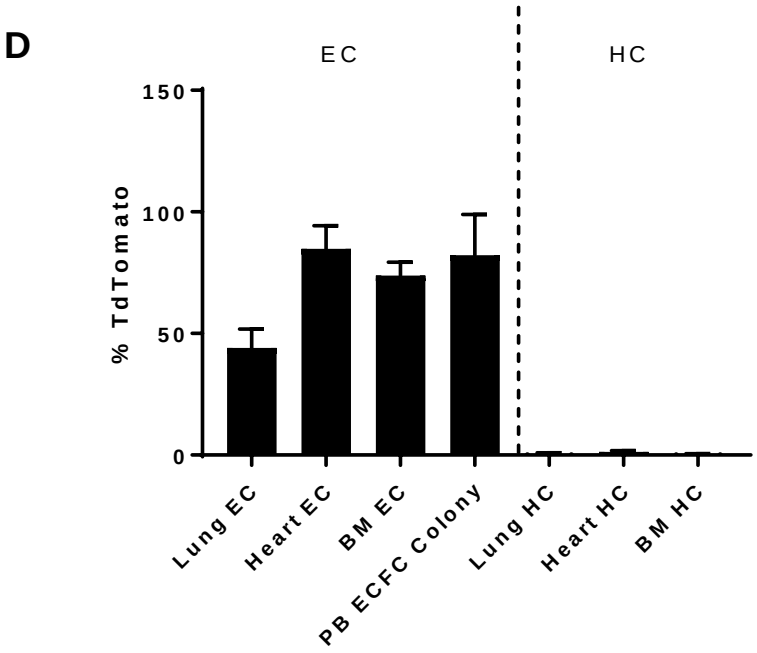
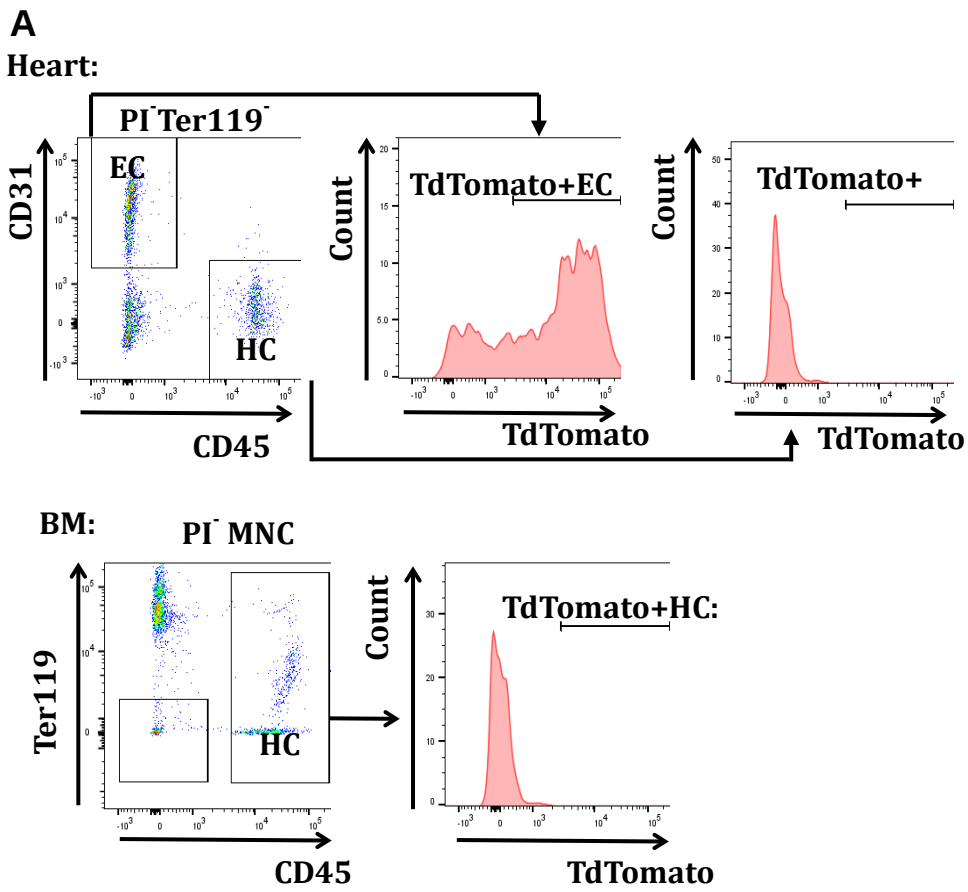
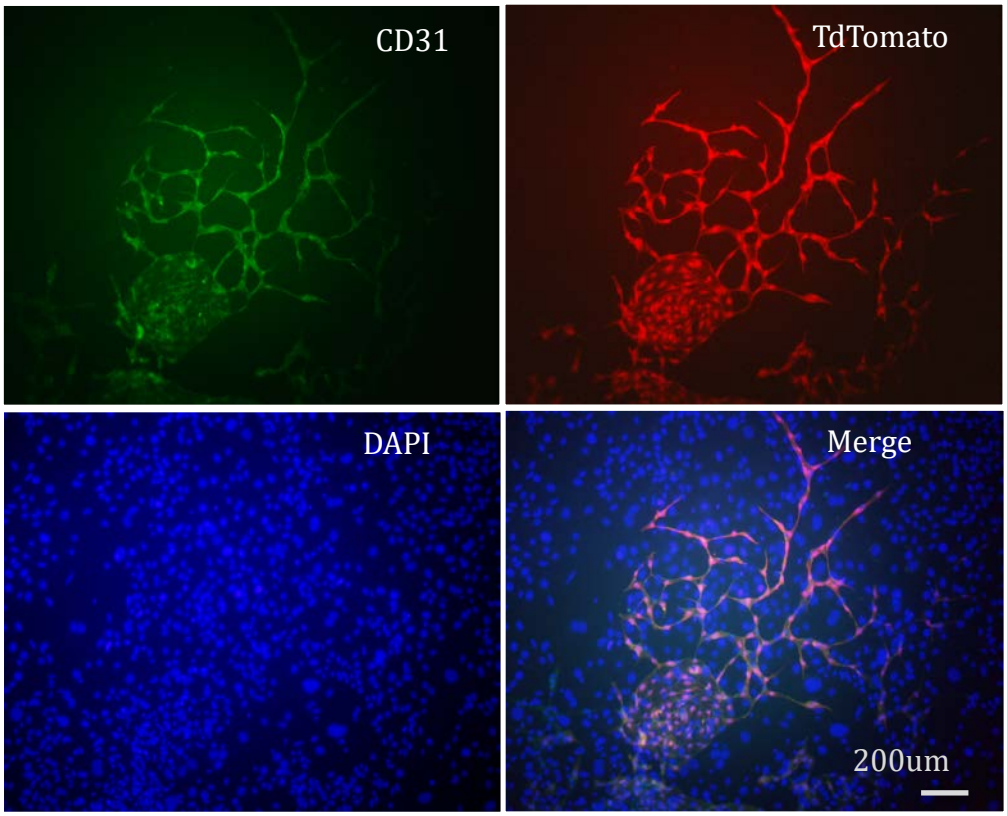
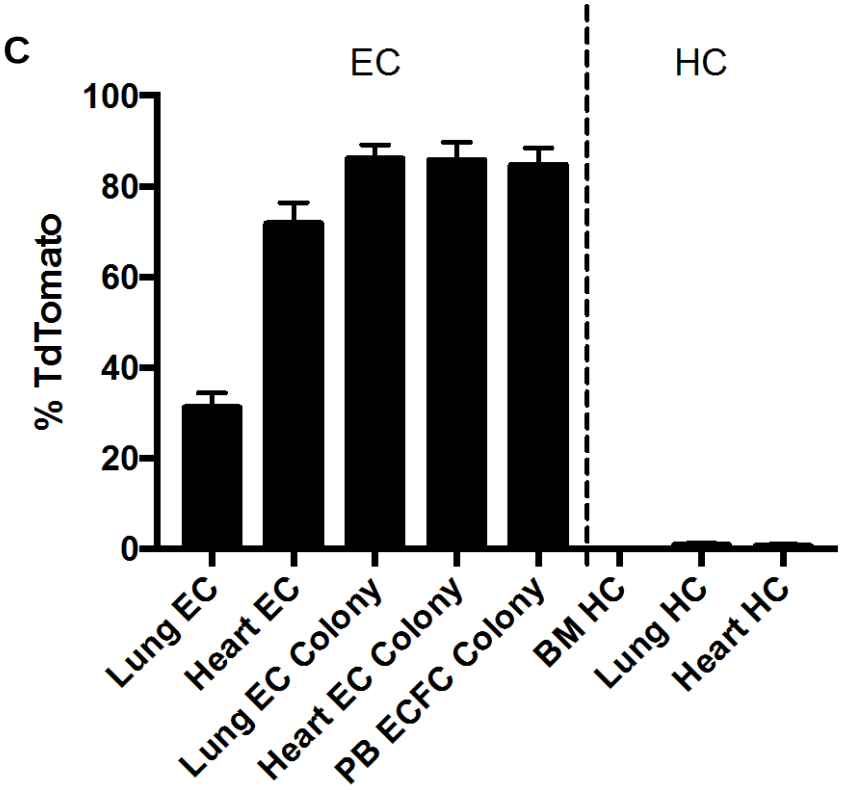


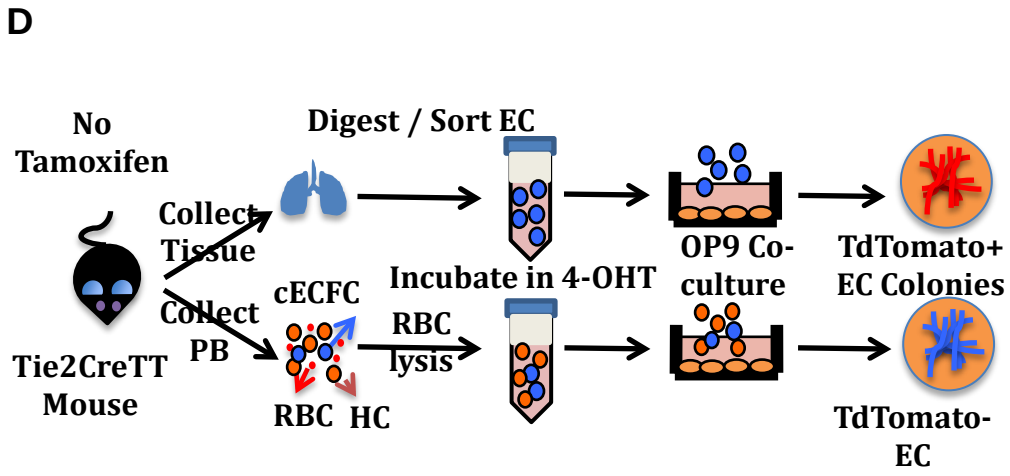
Figure III. 5. Lineage tracing using Cdh5TT mice shows cECFC are derived from vascular endothelial cells. (A). Schematics of lineage tracing using Cdh5TT mice (B). Flow cytometry analysis of postnatal day 4 Cdh5TT mice that receive daily 50mg/g tamoxifen injection at P0-P1. (D). cECFC colonies derived from peripheral blood of Cdh5TT mice. 52 out of 56 circulating ECFC Colonies were TdTomato+. (D). Quantitation of labeling efficiency of endothelial lineage and hematopoietic lineage cells from Cdh5TT mice.

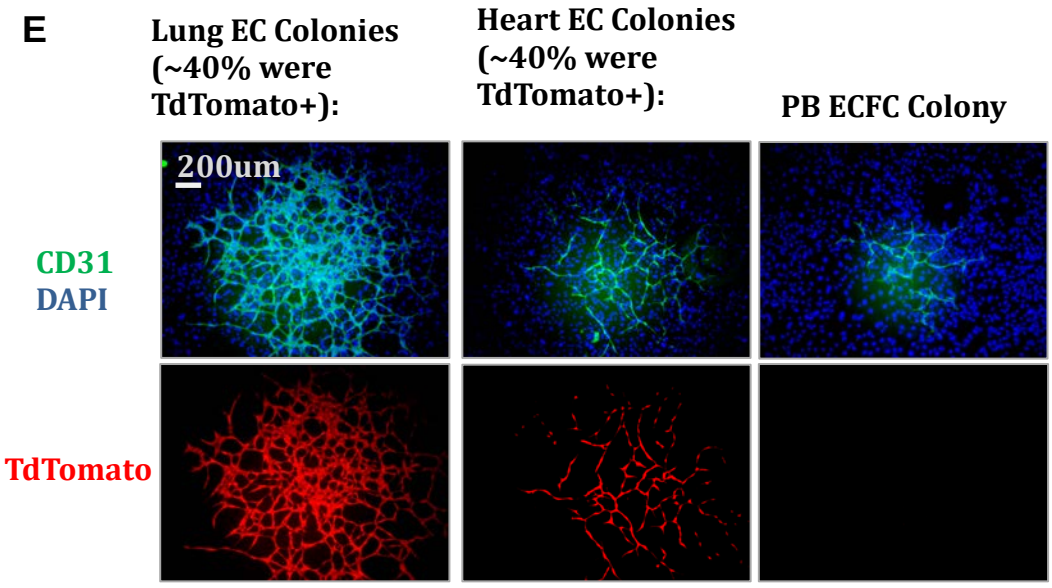

A
Heart:
PI-Ter119-
EC
HC
CD31
CD45
Count
TdTomato+EC
TdTomato
Count
TdTomato+
TdTomato
BM:
PI- MNC
Ter119
HC
CD45
Count
TdTomato+HC:
TdTomato

B




C
EC
HC
100
80
60
40
20
0
% TdTomato
Lung EC
Heart EC
Lung EC Colony
Heart EC Colony
PB ECFC Colony
BM HC
Lung HC
Heart HC


D
No Tamoxifen
Tie2CreTT Mouse
Collect Tissue
Collect PB
Digest / Sort EC
cECFC
RBC lysis
RBC
HC
Incubate in 4-OHT
OP9 Co-culture
TdTomato+ EC Colonies
TdTomato- EC


E
Lung EC Colonies (~40% were TdTomato+):
Heart EC Colonies (~40% were TdTomato+):
PB ECFC Colony
200um
CD31
DAPI
TdTomato

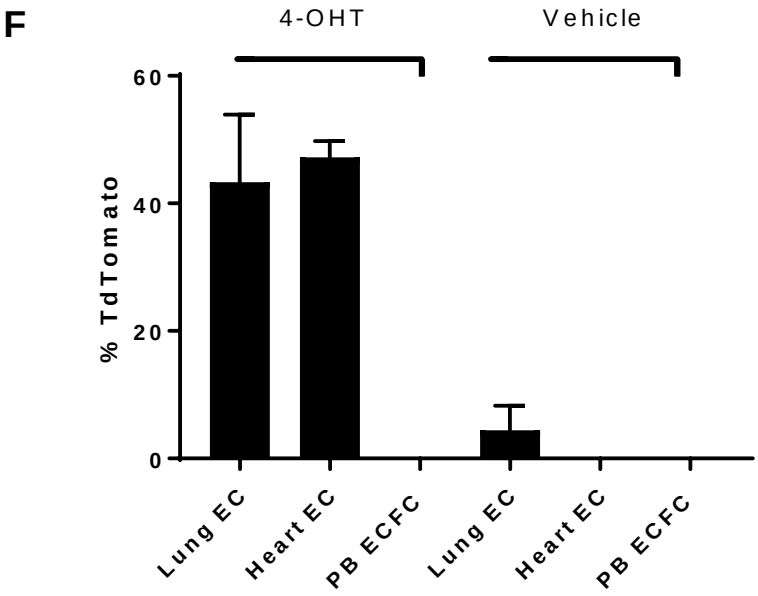
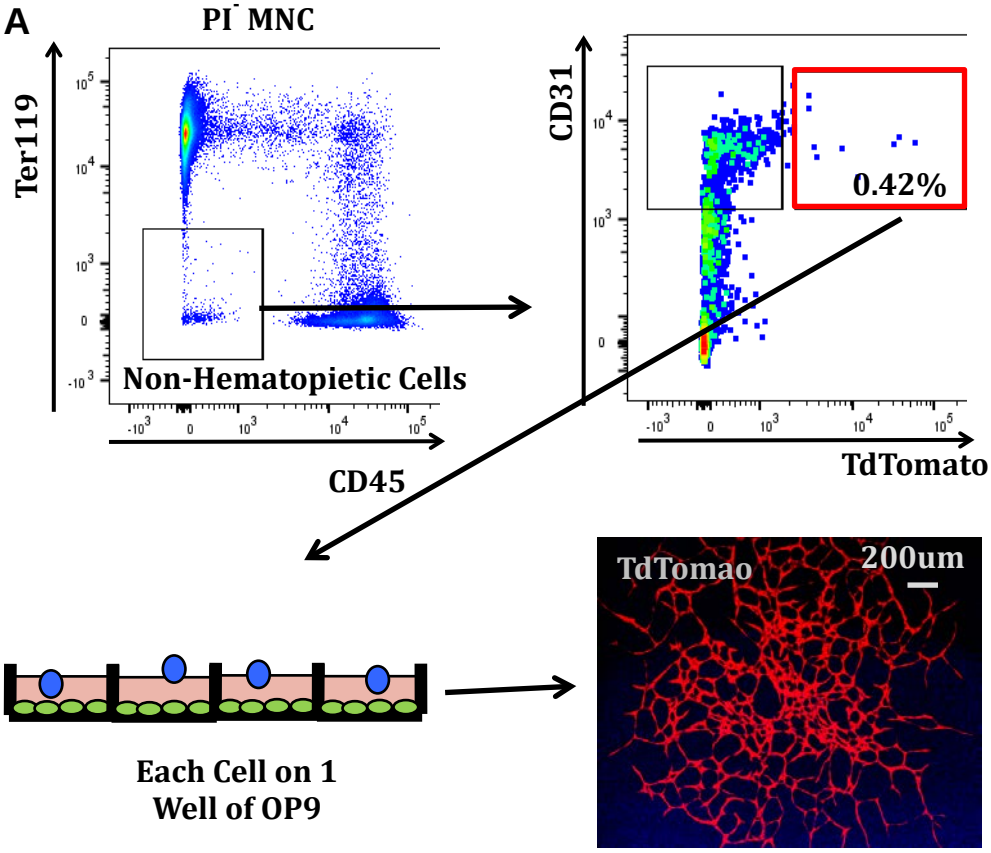

F
4-OHT
Vehicle
% TdTomato
60
40
20
0
Lung EC
Heart EC
PB ECFC
Lung EC
Heart EC
PB ECFC

Figure III. 6. Lineage tracing using Tie2TT mice shows cECFC are derived from vascular endothelial cells. (A). Flow cytometry analysis of postnatal day 4 Tie2TT mice that receive daily 50mg/g tamoxifen injection at P0-P1. (B). cECFC colonies derived from peripheral blood of Tie2TT mice. 155 out of 177 circulating ECFC colonies from 15 pups were TdTomato+. (C). Quantitation of labeling efficiency of endothelial lineage and hematopoietic lineage cells from Tie2TT mice. (D). Schematic of *in vitro* 4-OHT activation of Tie2-expressing ECFC. (E). Representative pictures of EC colonies derived from circulating or resident ECFC after *in vitro* 4-OHT treatment. (F). Quantitation of (E)

Human cECFC cannot be prospectively isolated (lack a unique antigen) and this hampers pursuit of distinguishing features as culturing the cells may change their properties. Since the majority of cECFC can be labeled by TdTomato in Tie2CreTT mice, we gave tamoxifen injections on P0 and P1, to identify $CD45^{-}Ter119^{-}CD31^{+}TdTomato^{+}$ MNC from P2 peripheral blood by flow cytometry (Figure III. 7A). When plated at a clonal level, cECFC gave rise to EC colonies in 17% of the wells (Figure III. 7A, B). In contrast, only 1% of $CD45^{-}Ter119^{-}CD31^{+}TdTomato^{+}$ cells from P2 heart tissue and 0.1% from P2 lung tissue formed EC colonies (Figure III. 7B). Thus, the Tie2CreTT PB $CD45^{-}Ter119^{-}CD31^{+}TdTomato^{+}$ cell population in the bloodstream is highly enriched with cECFC. When examined through the AMNIS imaging flow cytometry system, some freshly isolated cECFC lacked CD144 expression in comparison to lung, heart, and BM resident EC that all expressed CD144 (Figure III. 8). All fresh cECFC uniquely expressed P-selectin glycoprotein ligand 1 (PSGL1) (Figure III. 8), an adhesion molecule known to be expressed by human cECFC, but not mature EC, and proven to facilitate cECFC adherence to injured vasculature *in vivo* (Hubert et al., 2014). Thus, cECFC display some differences in cell surface markers compared to resident EC.


A
PI- MNC
Ter119
Non-Hematopietic Cells
CD45
CD31
0.42%
TdTomato
TdTomao
200um
Each Cell on 1
Well of OP9

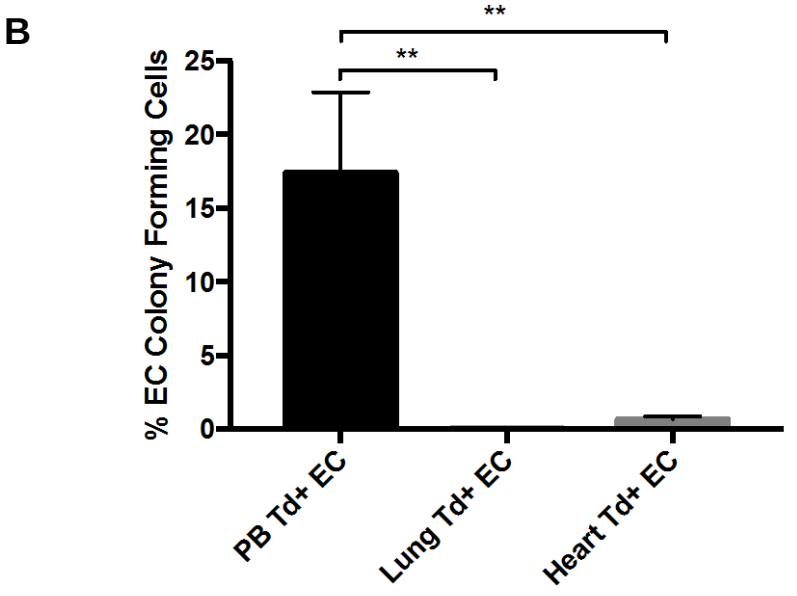

B
% EC Colony Forming Cells
25
20
15
10
5
0
**
**
PB Td+ EC
Lung Td+ EC
Heart Td+ EC

Figure III. 7. Single CD45$^-$Ter119$^-$CD31$^+$TdTomato$^+$ cells from the peripheral blood of Tie2CreTT mice have EC colony forming potential. (A). Schematic of single cell colony forming assay using P2 Tie2CreTT mice peripheral blood CD45$^-$Ter119$^-$CD31$^+$TdTomato$^+$ cells. Lower right panel shows a representative picture of a single CD45$^-$Ter119$^-$CD31$^+$TdTomato$^+$ cell formed colony. (B). Quantitation of the frequency of colony forming cells in P2 Tie2CreTT mice peripheral blood (PB), lung, or heart derived CD45$^-$Ter119$^-$CD31$^+$TdTomato$^+$ cells. ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

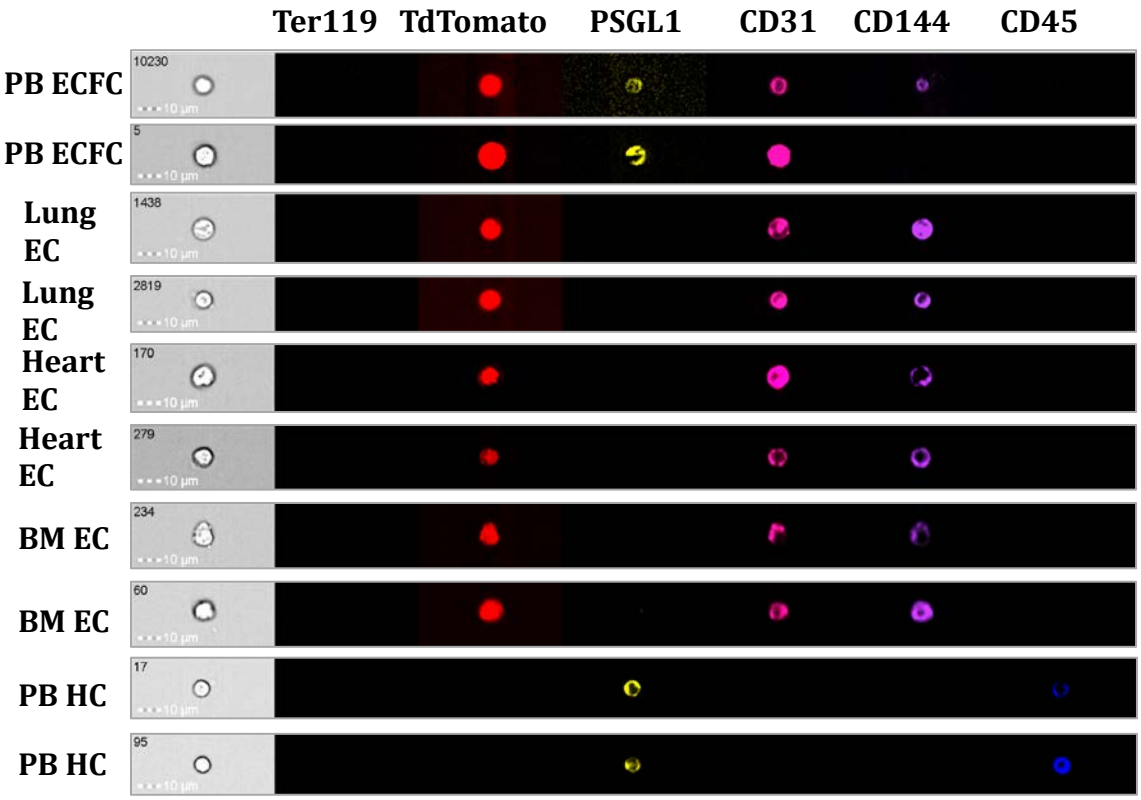
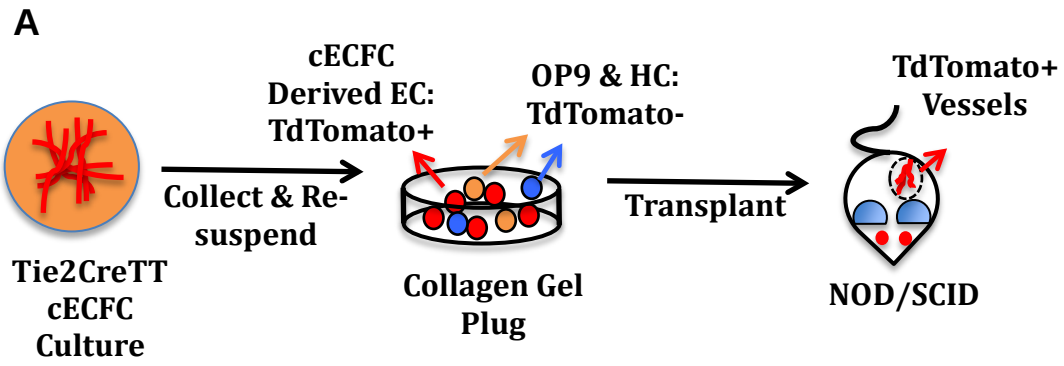


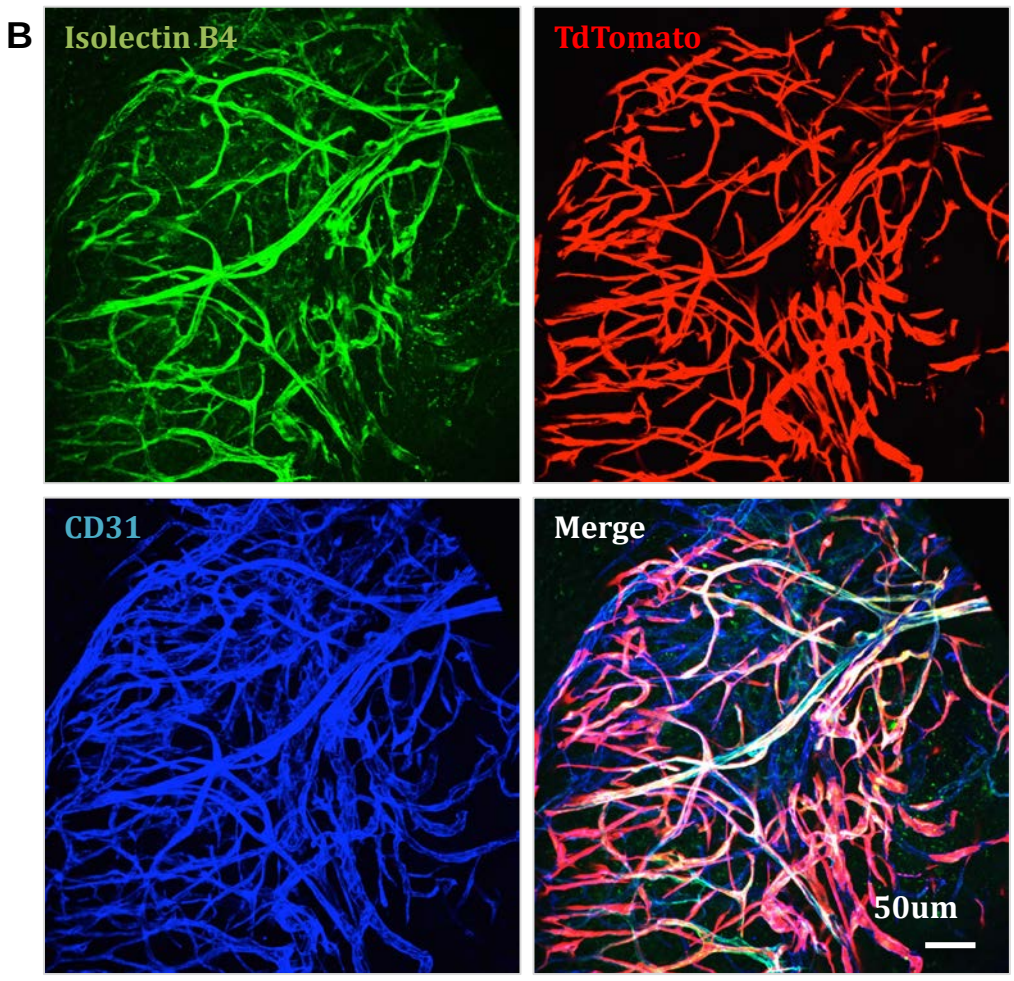
Figure III. 8. Imaging flow cytometry (AMNIS) analysis of surface marker expression on P2 Tie2CreTT mice peripheral blood (PB), lung, heart, bone marrow (BM) derived $CD45^{-}Ter119^{-}CD31^{+}TdTomato^{+}$ Single endothelial cells (EC) and PB $CD45^{+}$ hematopoietic cells (HC).

Murine cECFC represent circulating endothelial stem cells (CESC) that can form functional blood vessel *in vivo* and have the ability to self-renew

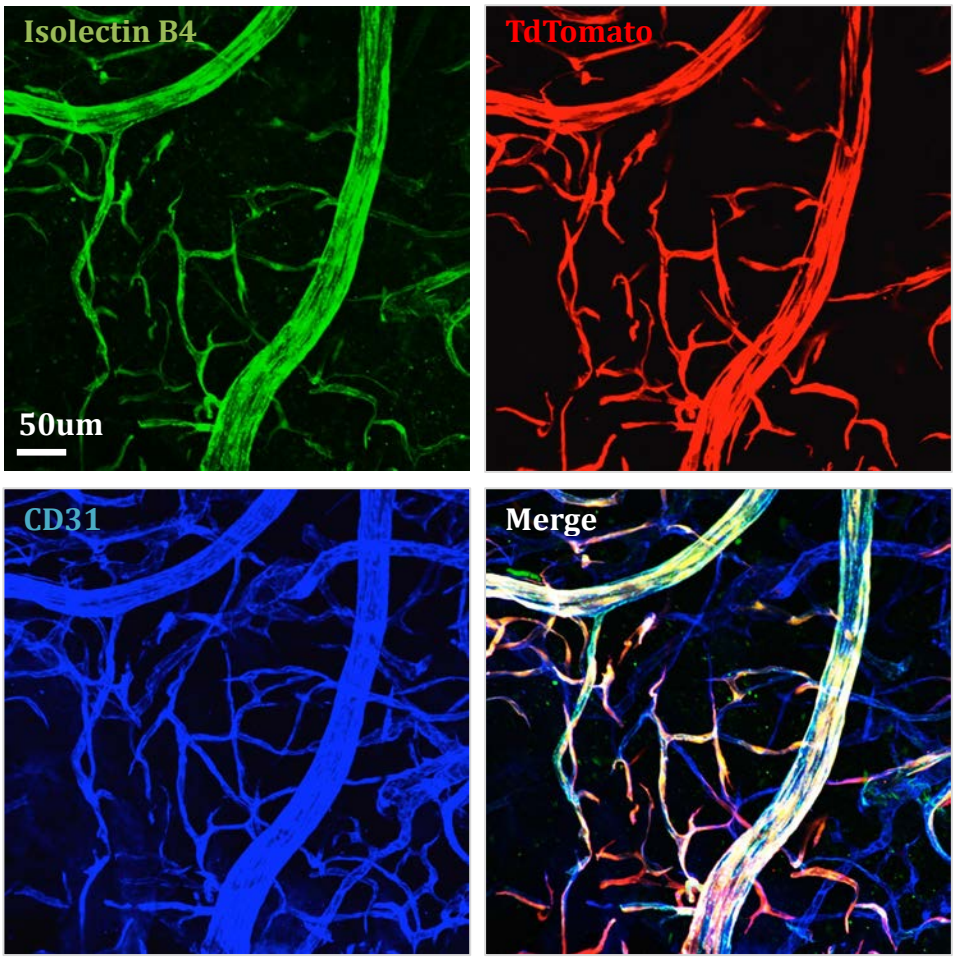
Murine cECFC, similar to human cECFC, can grow robustly *in vitro* and display a hierarchy of proliferative potential. These features made us curious about the potential of murine cECFC to form blood vessels *in vivo* like human cECFC.

In Tie2CreTT neonatal PB culture, only cECFC derived cells were TdTomato^{+} while OP9 and other donor derived cell types, like hematopoietic cells, remained TdTomato- (Figure III. 1). We transplanted type 1 collagen gel plugs mixed with Tie2CreTT cECFC cultured cells into NOD/SCID mice (Figure III. 9A). Within 2 weeks after implantation, inosculated donor blood vessels could be detected from all the retrieved gels (Figure III. 9B, C). These donor-derived vessels were perfused (confirmed by i. v. injection of fluorescein labeled Isolectin-B4, [IB4] Figure III. 9B, C), and were stable for up to 10 months (Data not shown). Clonal progeny of a single cECFC formed functional blood vessels *in vivo* after transplantation (Figure III. 9D), confirming that cECFC have prominent vessel forming potential.


A
cECFC
Derived EC:
TdTomato+
OP9 & HC:
TdTomato-
TdTomato+
Vessels
Collect & Re-
suspend
Transplant
Tie2CreTT
cECFC
Culture
Collagen Gel
Plug
NOD/SCID


B
Isolectin B4
TdTomato
CD31
Merge
50um

C



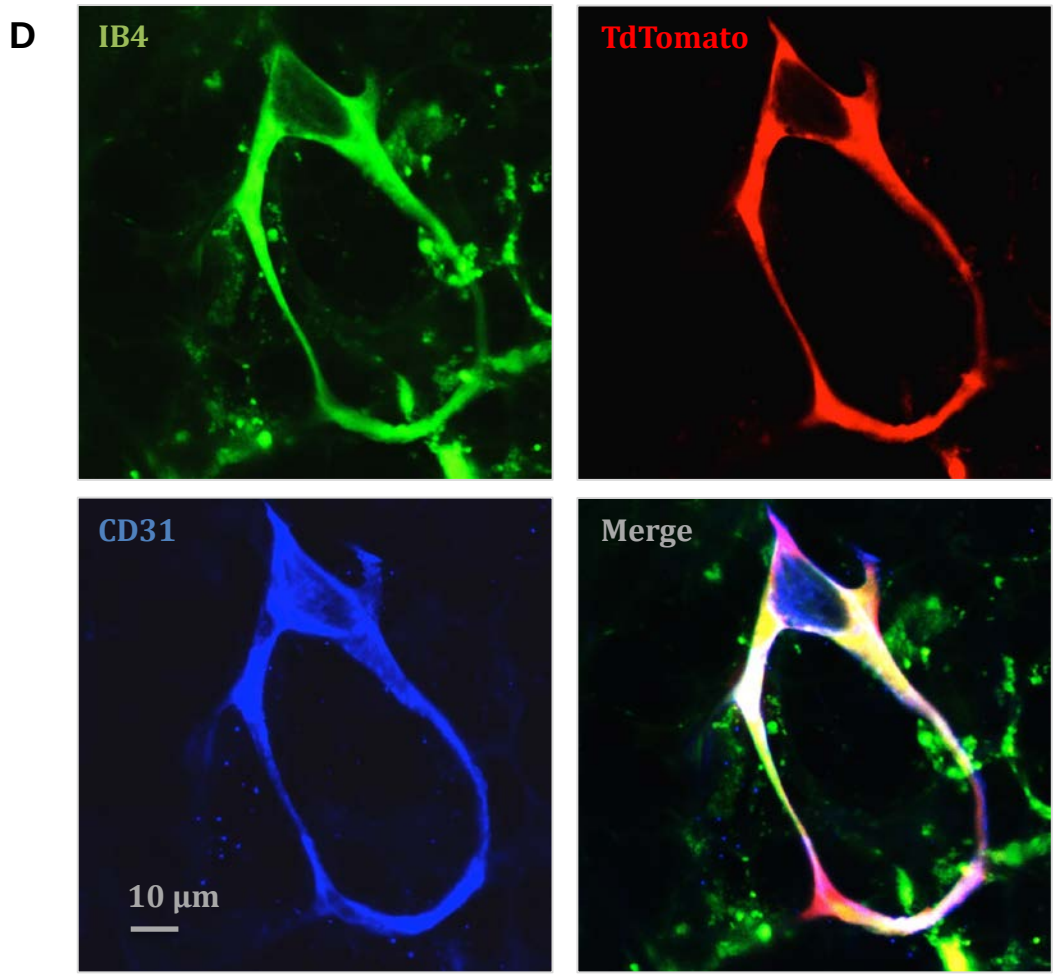


Figure III. 9. Tie2CreTT cECFC derived cells can form functional blood vessels *in vivo* after transplantation. (A). Schematic of collagen plug transplantation assay using P2 Tie2CreTT mice peripheral blood cECFC derived cells. (B, C). 2 weeks after transplantation, cECFC derived blood vessels (TdTomato+) are inosculated with host vasculatures (shown by the labeling of isolectin B4). (D). A single Tie2CreTT cECFC derived blood vessel *in vivo* 4 months after transplantation.

In addition, when P3 Tie2CreTT PB cECFC containing gels were recovered after 3 weeks of implantation, digested, and the recovered EC re-plated on OP9, 7.33±4.04 secondary TdTomato$^+$ EC colonies were observed from each gel (Figure III. 10). These results confirm that cultured cECFC can undergo postnatal vasculogenesis after transplantation, and not only form vessels but give rise to vascular resident ECFC (vECFC) that can produce numerous EC progeny. This ability for secondary ECFC production has also been confirmed in human cord blood cECFC (Figure III. 11). Thus, not only can cECFC form *in vitro* colonies and *in vivo* blood vessels, they can also self-renew *in vivo* by retaining their proliferative potential and therefore represent circulating endothelial stem cells (CESC).

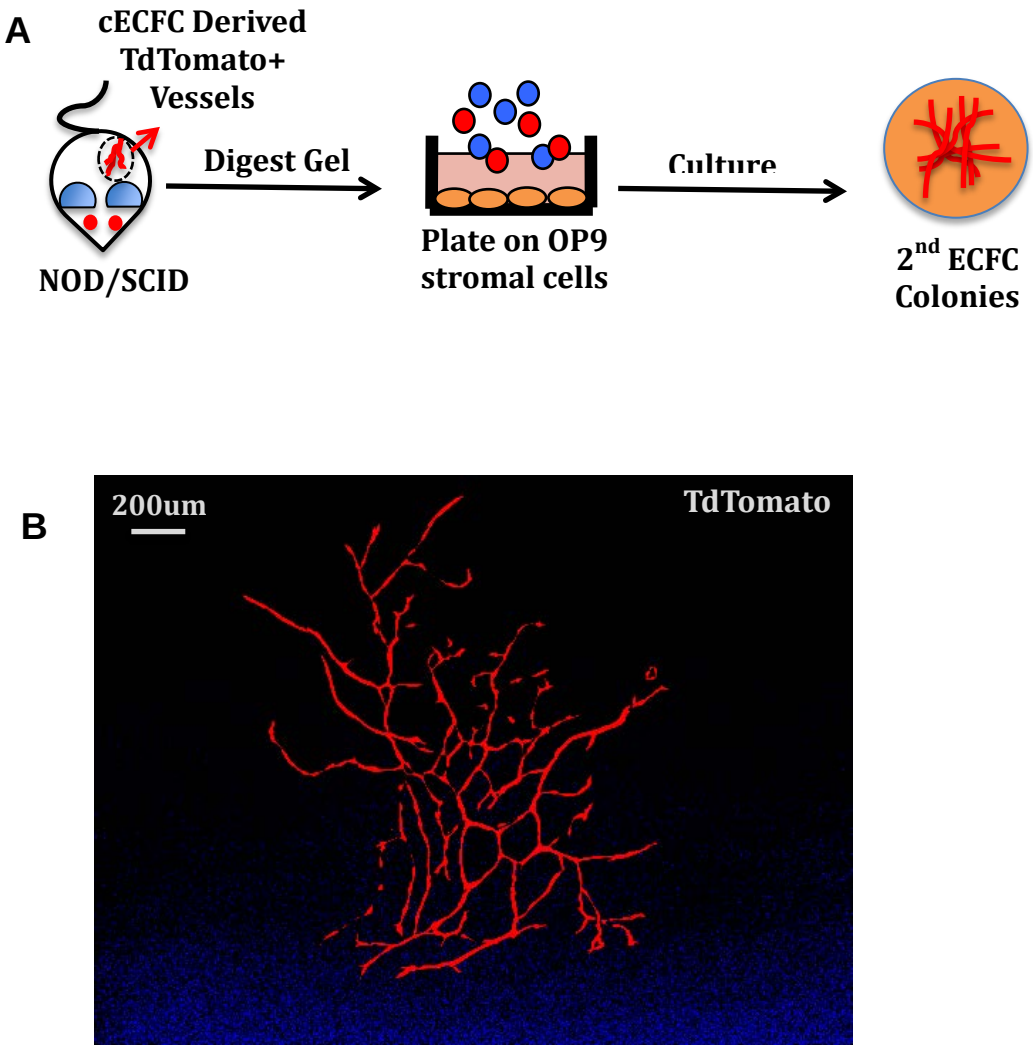


Figure III. 10. Tie2CreTT cECFC have the ability to self-renew. (A). Schematic of the assay that test the self-renewal potential of cECFC. (B). A representative picture of a TdTomato^{+} secondary colony from Tie2CreTT mice derived cECFC fromed gel 3 months after transplantation.

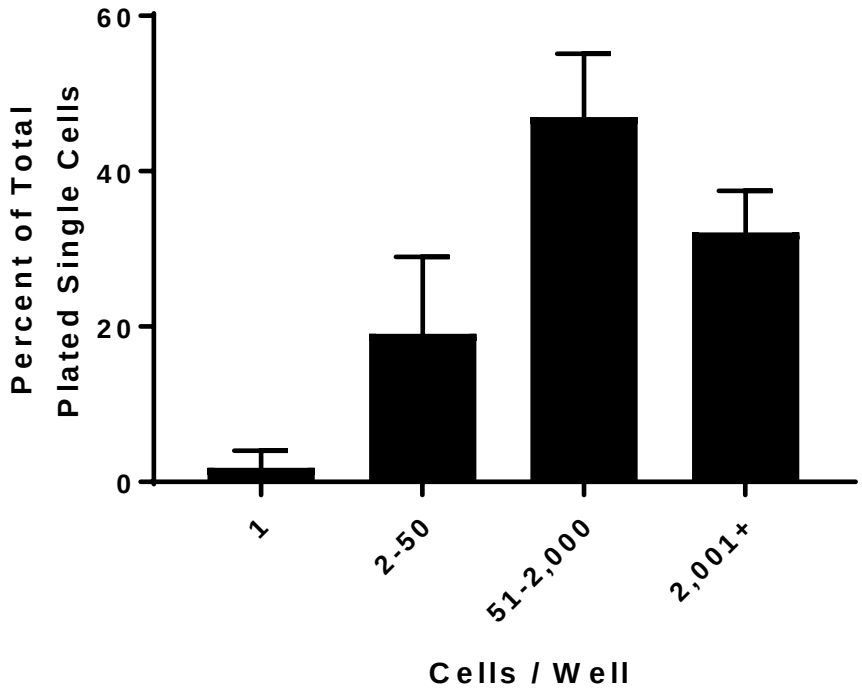
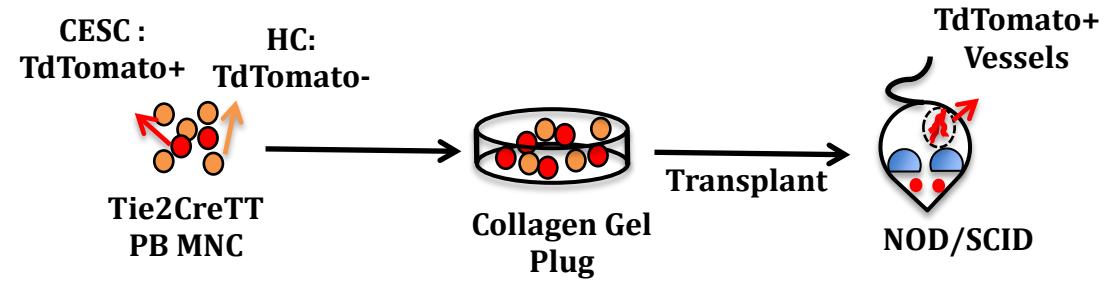


Figure III. 11. 2nd EC culture grew from human cord blood cECFC derived blood vessels show a hierarchy of proliferative potential in single cell colony forming assay.

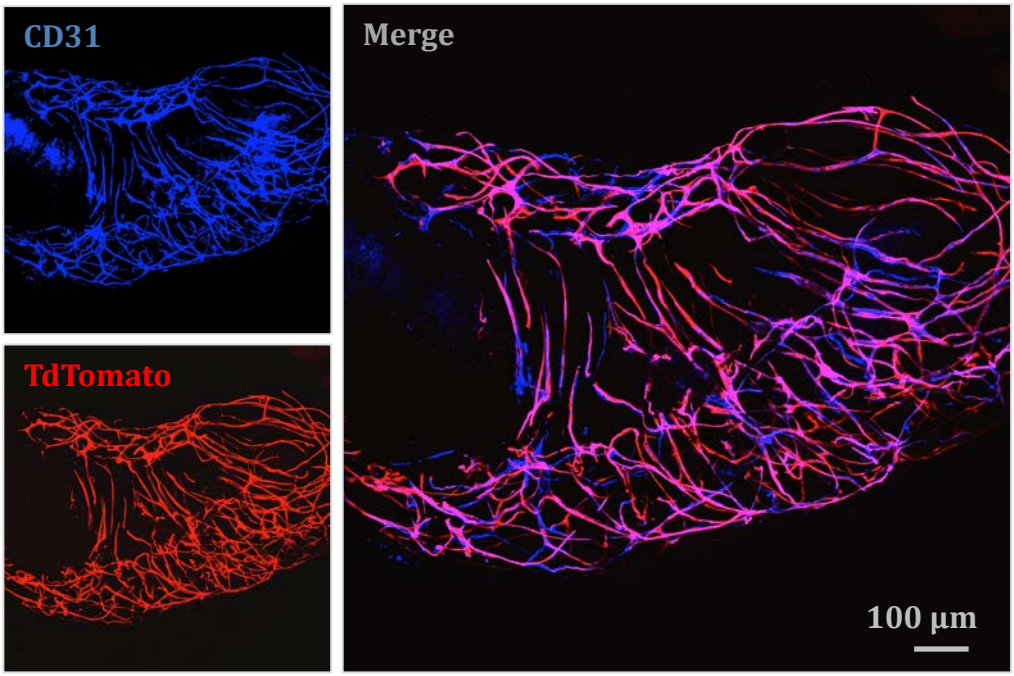
To assess if uncultured CESC possess vessel forming potential *in vivo*, we implanted collagen gels with freshly isolated, uncultured PB MNC from neonatal Tie2CreTT mice into NOD/SCID mice (Figure III. 12A) and inosculated donor TdTomato$^+$ blood vessels were identified from all gel implants (Figure III. 12B, C).

Furthermore, we also transplanted uncultured human cord blood CD34$^+$CD45$^-$ cells, which are enriched with CESC (Mund et al., 2012; Timmermans et al., 2007), into NOD/SCID mice and after 2-4 weeks, robust functional human blood vessels were identified from all the retrieved gels (Figure III. 13). Thus, for the first time, both murine and human CESC have been shown to display the ability to directly form functional vessels via postnatal vasculogenesis *in vivo* without prior culture, disputing some claims that CESC are a cell type that represents an *in vitro* culture artifact.

A


CESC :
TdTomato+
HC:
TdTomato-
Tie2CreTT
PB MNC
Collagen Gel
Plug
Transplant
TdTomato+
Vessels
NOD/SCID

B


CD31
TdTomato
Merge
100 µm

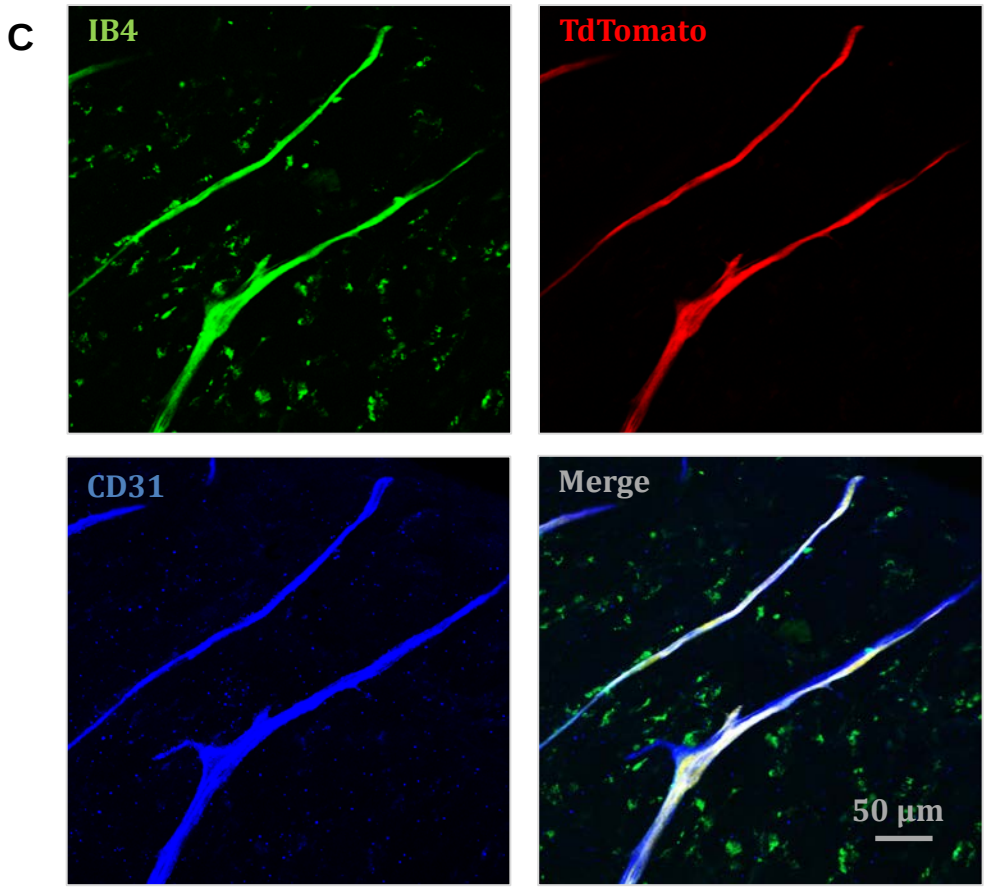


Figure III. 12. Uncultured Tie2CreTT CESC can form functional blood vessels *in vivo* after transplantation. (A). Schematic of collagen plug transplantation assay using uncultured P2 Tie2CreTT mice peripheral blood which contain TdTomato^{+} CESC. (B, C). 4 weeks after transplantation, uncultured CESC derived blood vessels (TdTomato^{+}) are inosculated with host vasculatures (shown by the labeling of isolectin B4).

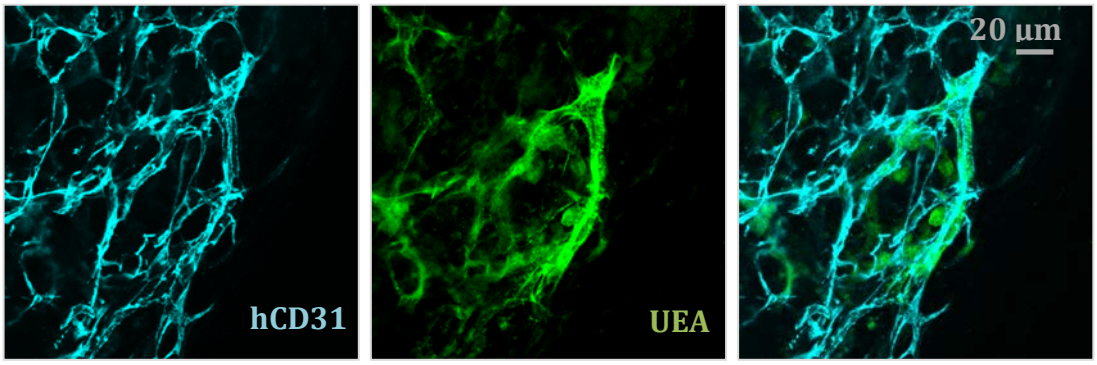


Figure III. 13. Uncultured human cord blood $CD34^{+}CD45^{-}$ cells contain CESC that can form functional blood vessels *in vivo* after transplantation. UEA1, Ulex Europaeus Agglutinin I, shows the perfusion of human CESC derived vessels.

CESC are labeled by Abcg2

We have shown in Chapter I that resident VESC are marked by drug transporter Abcg2. Next we tested if the expression of Abcg2 also labels CESC in the blood stream. P1 PB as well as heart / lung EC from ABCG2TT mice were collected and incubated in 4-OHT for 8 hours before plated on OP9. After 7 days, about 14 out of 66 of CESC colonies from 9 pups were labeled by TdTomato (Figure III. 14), proving that CESC also express *Abcg2*.

Additionally, human cord blood mononuclear cells were also separated as $ABCG2^+$ and $ABCG2^-$ fraction by MACS. After culture, $ABCG2^+$ cord blood $CD34^+CD45^-$ cells are more enriched with EC colony forming cells (Figure III. 15). Thus like murine CESC, human CESC also express ABCG2.

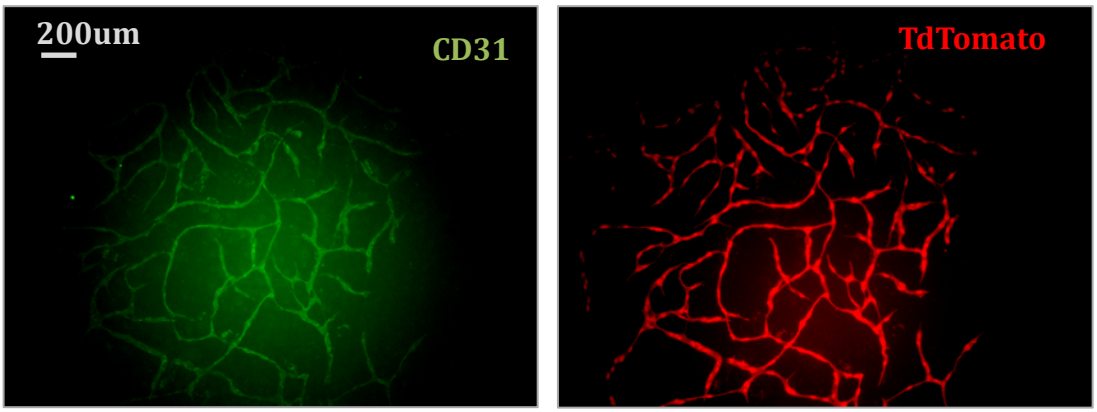
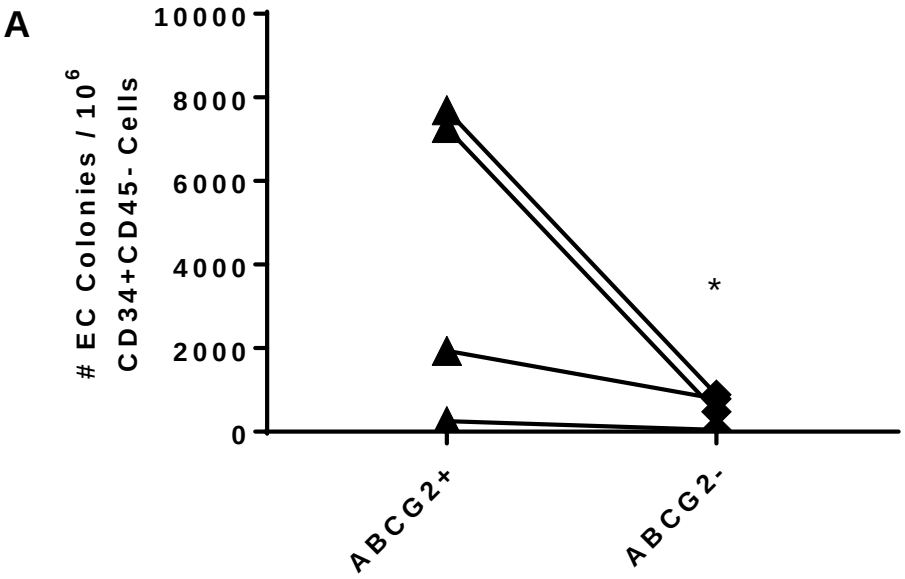


Figure III. 14. In vitro 4-OHT labeled CESC from ABCG2TT mice formed TdTomato$^+$ EC colonies (14 out of 66 colonies from 9 pups).


A
10000
8000
6000
4000
2000
0
EC Colonies / 10^6 CD34+CD45- Cells
*
ABCG2+
ABCG2-

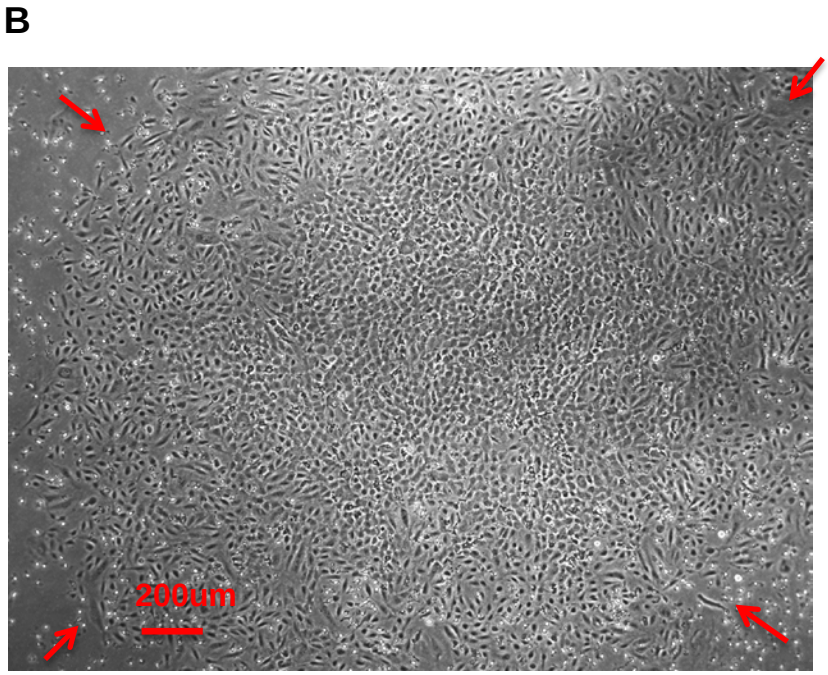
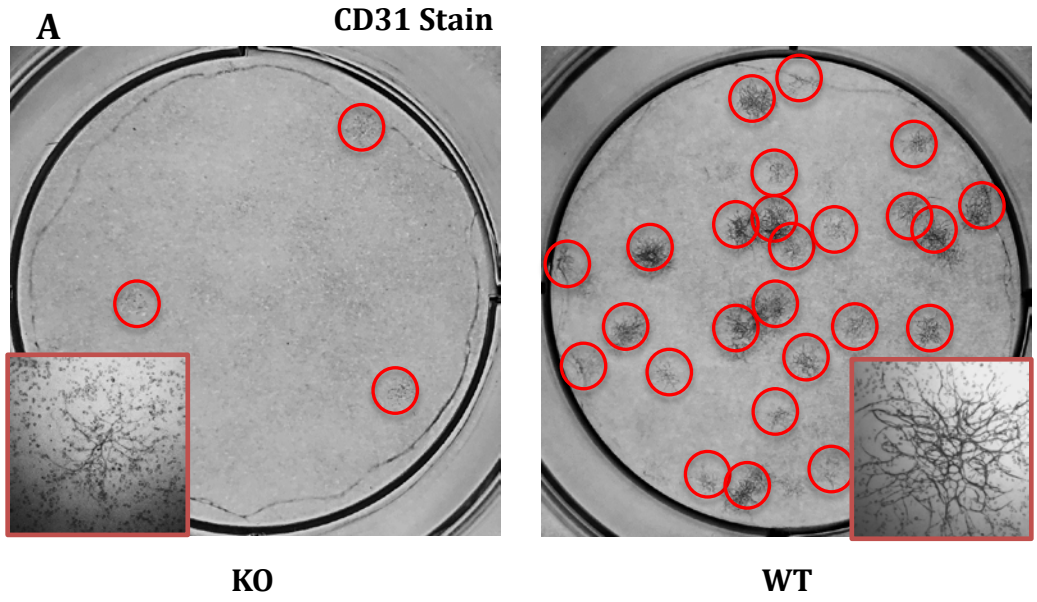
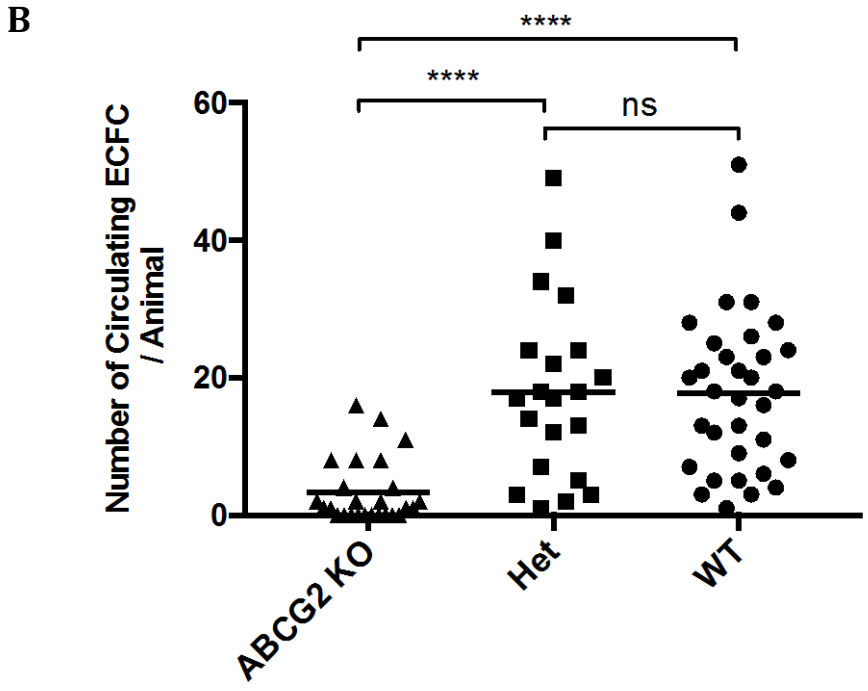

B
200um

Figure III. 15. Human cord blood CESC are labeled by ABCG2 (A). Frequency of EC colony forming cells in $ABCG2^+$ and $ABCG2^-$ $CD34^+CD45^-$ cord blood cells. (B). A representative picture of a EC colony derived from $ABCG2^+$ human cord blood CESC 10 days after culture. *, $p<0.05$.

The production of CESC is Abcg2 dependent

We next used *Abcg2* knockout (KO) mice to discern if *Abcg2* is important for the production/maintenance of CESC. We have proven that the presence of *Abcg2* is crucial for the maintenance of resident VESC in Chapter II. We also isolated PB from P1 wild type and *Abcg2* knockout (KO) mice and cultured them on OP9. Notably, loss of *Abcg2* almost completely abolished the production of CESC (Figure III. 16A, B). Since *Abcb1a* (Mdrr1a) and *Abcb1b* (Mdr1b) were also expressed in SP ECFC and both transcripts increased in expression in *Abcg2* KO mice (Figure II. 3, II. 9), we administered an inhibitor of *Abcb1a* and *Abcb1b* transporter function (Tetrandrine) to newborn pups via intraperitoneal injection from P0-P3. As the result, the emergence of CESC in the *Abcg2* KO mouse pups was even more significantly hampered (Figure III. 16C). These data confirm that *Abcg2* plays a critical role in not only the maintenance of VESC, but also the emergence CESC.


A
CD31 Stain
KO
WT


B
****
****
ns
Number of Circulating ECFC / Animal
60
40
20
0
ABCG2 KO
Het
WT

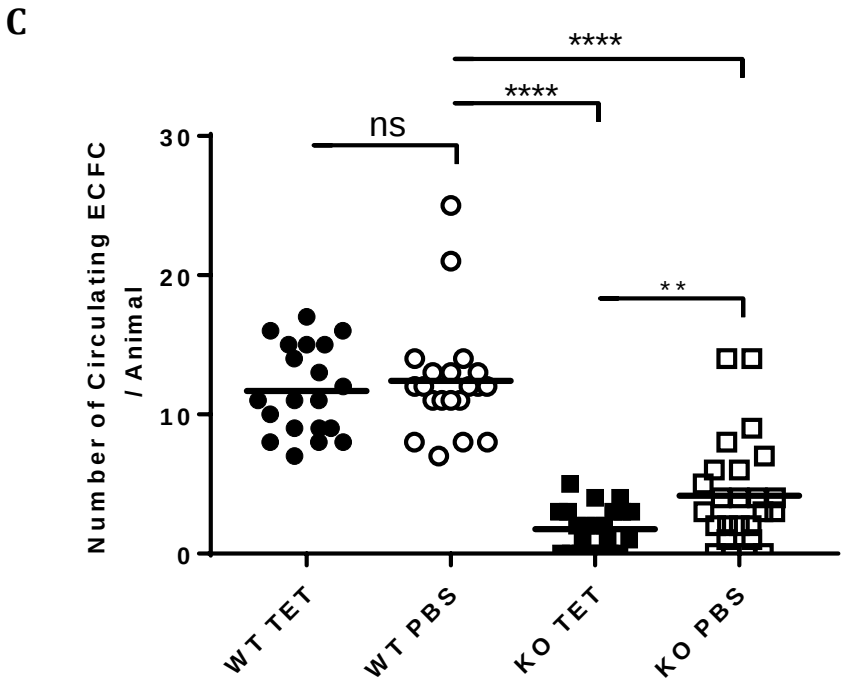


Figure III. 16. Abcg2 is crucial for the production of CESC. (A). Representative picture of CESC colonies derived from the peripheral blood of 1 P1 Abcg2 knockout (KO) and wild type FVB (WT) mouse. (B). Quantitation of the number of CESC in P1 Abcg2 knockout, heterozygous, or wild type FVB (WT) mice. (C). Quantitation of the number of CESC in P4 Abcg2 knockout (KO) or wild type FVB (WT) mice that received tetrandrine or PBS injection at P1-P3. ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

4. Discussion

In sum, we show that murine vascular EC derived CESC give rise to a hierarchy of endothelial colonies *in vitro*, form functional blood vessels (that contain resident ECFC) *in vivo*. We also showed that fresh human and murine CESC, without *in vitro* culture, can form robust blood vessels after transplantation and though CESC are relatively rare in the blood, their potential contribution to vessel formation and repair cannot be underestimated. Indeed, in our study, the progeny of a single cultured murine CESC, gave rise to a robust functional blood vessel network upon transplantation in a 400X400um area (Figure III. 9D) and the donor-derived vessels persisted for up to 4 months.

In many species, CESC are more enriched during development and can be rapidly released after injury (Huang et al., 2007; Ingram et al., 2004; Shelley et al., 2012). However the molecular pathways that control CESC production are still largely unidentified. In this study, we proved that A*bcg2*, which labels both VESC and CESC, is a major molecular determinant of CESC production. Furthermore, our identification of this set of tools to prospectively isolate CESC from the blood will give new avenues to identify the critical roles played by CESC in vascular development, homeostasis, and repair; all with translational potential to human subjects.

Chapter IV

Ongoing Experiments and Future Directions

1. Gene expression analysis for *Abcg2*-expressing VESC

We have proved that in neonatal and adult mice, *Abcg2*-expressing EC represent VESC that can form EC colonies in vitro, form functional blood vessels *in vivo*, and contribute to blood vessel growth during development and tissue regeneration. These cells are intrinsically different from mature EC which only have limited *in vitro* colony forming potential and restricted ability to form blood vessels *in vivo*. Various cardiovascular diseases are linked with abnormal functions of vascular progenitor / stem cells (Baker et al., 2012; Borghesi et al., 2009; Chang et al., 2017; Prasain et al., 2014). Thus, to understand the underline mechanism of these diseases, it will be meaningful to understand the difference between the gene expression patterns of VESC and mature EC.

We have injected 50mg/g body weight tamoxifen to the P0 neonatal ABCG2TT pups and isolated TdTomato$^+$ EC, which represent *Abcg2*-expressing VESC, and TdTomato$^-$ mature EC, to perform transcriptome analysis between these two groups. We will use this data to understand the pattern of gene expression of VESC, look for additional cell surface markers that are specific for VESC, and study the mechanism of VESC's self-renew and differentiation.

Because VESC among different tissue vascular beds show some differences in colony forming potential and spatial localization, and the frequency of VESC in each tissue varies, we also collected TdTomato$^+$ and TdTomato$^-$ EC from the lung and skeletal muscle of P1 ABCG2TT pups for RNA sequencing to study the tissue specificity of VESC. This experiment will provide information on the intrinsic specificity of VESC in each tissue and explain the difference of vessel regeneration ability among different tissues.

In our study using adult ABCG2TT mice, we have found that *Abcg2*-expressing VESC also exist in adult tissues, and they possess the potential to participate vessel regeneration after injury. Compared to neonatal tissues, adult organs are more quiescent and require less proliferation for repair or replacement at homeostasis. Thus adult VESC may differ from neonatal VESC in terms of cell cycle regulation, proliferation and functions. Indeed, adult lung and heart contain less frequent VESC than neonatal tissues, and adult organs have lower frequency of EC colony forming cells compared with developing tissues. However these quiescent adult EC progenitor / stem cells can be activated and proliferate during physiological and pathological conditions (Alvarez et al., 2008; Ding et al., 2011; Nishimura et al., 2015). To understand the mechanism of VESC maintenance and understand the difference between VESC in developing and homeostatic tissues, we also isolated TdTomato$^+$ VESC and TdTomato$^-$ mature EC from 7 weeks old adult ABCG2TT mice that received tamoxifen injection 24 hours before the collection to run gene expression analysis.

In future studies, we will perform single cell RNAseq analysis using the Smart2 method to probe individual *Abcg2*-expressing VESC to investigate the differences among the transcriptome of VESC residing in arteries, veins, and capillaries. By looking for EC with similar gene expression patterns as *Abcg2*-expressing VESC, this experiment will also help us to find other EC stem / progenitor populations in the vasculature.

2. The effect of the depletion of VESC

In our studies, *Abcg2*-expressing VESC showed significant contribution to the vessel growth in live animals during development and tissue regeneration. However it is still not clear whether if other cell populations, like cells from other lineages, or mature EC, can contribute to blood vessel growth / regeneration without VESC. To answer this question, we crossed Abcg2ERTCre mice with $FoxF1^{flox}$ mice to make $ABCG2CreFoxF1^{f/f}$ mice. It is known that FoxF1 is crucial to the maintenance of vascular EC and mice lacking FoxF1 in EC die in 2 weeks due to vascular defects (Ren et al., 2014). Thus, after tamoxifen was given to $ABCG2CreFoxF1^{f/f}$ mice at different development stages, *Abcg2*-expressing VESC, as well as EC progeny derived from *Abcg2*-expressing VESC, would be depleted from the blood vessels due to the lack of FoxF1. In the developing mice, a defect in the growth of vasculature is expected and can be detected through the morphology of different tissues, as well as immunohistochemistry and flow cytometry analysis. In adult mice, because most of vascular EC in homeostatic tissues are quiescent, and the frequency of *Abcg2*-expressing VESC is relatively low compared with neonatal mice, depletion of these

cells may have less dramatic effect than in developing mice. However, since *Abcg2*-expressing VESC actively participate vessel regeneration after tissue injury, adult ABCG2CreFoxF1$^{f/f}$ mice that received tamoxifen injection may show impaired vascular recovery in various experimental vessel injury models like hind limb ischemia and myocardial infarction.

As an alternative approach, we will also cross Abcg2ERTCre mice with ROSA diphtheria toxin receptor mice (DTR) to make ABCG2DTR mice. DTR mice has been widely used in combination with various lineage specific cre mice to precisely deplete cells that express a certain gene. When ABCG2DTR mice are given tamoxifen injection, all Abcg2 expressing cells, including VESC, will start to express DTR on the cell surface. All DTR expressing cells will subject to cell death upon the administration of diphtheria toxin. It is expected that the injection of diphtheria toxin in ABCG2DTR mice would cause the depletion of *Abcg2*-expressing VESC and lead to impaired vessel growth in the developing mice or compromised vessel regeneration in adult mice after vascular injuries.

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Publications

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Lin, Y et al., Embryonic stem cell-derived B cells are B-1 lineage and not an indicator for the presence of hematopoietic stem cells. Manuscript in preparation.

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2016. 19th International Vascular Biology Meeting. Boston, MA. Murine circulating endothelial colony forming cells are derived from resident endothelial cells and can participate in vessel formation *in vivo*. (Poster)

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